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Extracellular matrix (ECM) proteins are critical for differentiation of normal mammary epithelial cells, but pronounced changes in ECM occurs during breast cancer. We have determined that estrogen receptor (ER)-positive breast cancer cells (MCF-7 and T47D) cultured on laminin have reduced, or lack, estrogen responses. Estrogen-induced proliferation was significantly reduced on LM but did occur on collagens, fibronectin, poly-L-lysine or vitronectin. Laminin did not inhibit IGF-I or EGF induced proliferation, indicating that tumor cells on laminin do not become refractory to mitogens. An endogenous estrogen regulated protein, the progesterone receptor, was not significantly stimulated by estrogen on laminin; but was stimulated on collagen-I or fibronectin, indicating that laminin may inhibit ER binding or post-receptor events. However, ER content, ER binding and expression of ER splice variants was not different on different ECM proteins. Estrogen stimulation of ERE transcription was downregulated in MCF-7 cells cultured on laminin. MCF-7 cells injected into nude mice grew 5 times slower when coinjected with LM in response to E (when compared to Col I coinjection), and did not respond to antiestrogens. These studies demonstrate that ECM proteins may regulate estrogen action and anti-estrogen action which are critical in secondary treatment for breast cancer.

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## INTRODUCTION

While more than 60% of breast cancers are estrogen-receptor (ER) positive, only one third of estrogen receptor-positive tumors respond to endocrine therapy (Martin et al., 1994).

Understanding the mechanisms underlying the acquisition and loss of estrogen responsiveness in breast epithelium may be critical to the treatment of breast cancer since estrogen-responsive tumors have a better prognosis and are less likely to become metastatic than estrogen non-responsive tumors (Garcia et al., 1992; Moghazy et al., 1992). In the normal mammary gland, epithelial-stromal interactions are required for steroid-induced mammary gland growth and morphogenesis. However, breast carcinogenesis is often accompanied by pronounced changes in the stroma, termed desmoplasia, or 'the stromal reaction' (Ronnov-Jessen et al., 1996). In fact, desmoplasia is a prominent feature of infiltrating ductal carcinomas, which are the most common type of breast cancer. Since desmoplastic stroma has marked changes in both cellular composition and secreted proteins, it is likely that normal epithelial-stromal interactions, essential for estrogen-mediated proliferation, are altered during breast tumorigenesis.

Normal mammary epithelial cells in culture do not proliferate in response to ovarian steroids. However, mammary epithelial cells can proliferate in response to estrogen or progesterone when cultured with stromal cells or stromal-derived proteins (McGrath, 1983; Haslam, 1986; Xie and Haslam, 1997). In vivo, the surgical recombination of mature mammary stroma with immature mammary epithelium, transplanted into the mammary fat pad of an immature mouse, permits the precocious development of epithelial estrogen-inducible PR, required for branching morphogenesis (Haslam and Counterman, 1991). To determine if stromal tissue is required for estrogen-mediated mammary gland development, Cunha and colleagues (1997) performed

surgical tissue recombinations of wild-type (ER<sup>+/+</sup>) or ER knockout (ER<sup>-/-</sup>) mammary epithelium with ER<sup>+/+</sup> or ER<sup>-/-</sup> mammary stroma and transplanted them under the kidney capsule of athymic nude mice. Mammary gland ductal elongation and morphogenesis occurred when ER<sup>+/+</sup> stroma was combined with either ER<sup>-/-</sup> or ER<sup>+/+</sup> epithelium, but did not occur when ER<sup>-/-</sup> stroma was combined with either ER<sup>-/-</sup> or ER<sup>+/+</sup> epithelium. Collectively, these results demonstrate that normal mammary stroma is required for ovarian steroid mediated morphogenesis and proliferation of normal mammary epithelium.

Two major mechanisms by which stromal cells can influence epithelial cell behavior are by the secretion of growth factors and by altering the molecular composition of the extracellular matrix (ECM). In the normal mammary gland, stromal cells synthesize the major extracellular matrix proteins in the epithelial basement membrane (Keely et al., 1995). Our laboratory has previously demonstrated a requirement of specific ECM proteins, fibronectin (FN) or collagen IV (Col IV), for progesterone-induced proliferation of cultured mouse mammary epithelial cells (Xie and Haslam, 1997). Other ECM proteins are required for differentiation. For example, laminin (LM), a major ECM protein in the basement membrane, has been demonstrated to play a critical role in the lactational differentiation of normal mammary epithelium in vitro (Schmidhauser et al., 1990; Streuli et al., 1991). The effects of ECM proteins on estrogen action in breast cancer cells have not been determined.

In our previous annual report we had found that estrogen-induced cell proliferation in MCF-7 and T47D cells was modified by the attachment to ECM proteins. Specifically, laminin inhibited proliferation of both cell lines, while collagen I, collagen IV, fibronectin and vitronectin

permitted estrogen-induced proliferation. We also determined that estrogen-induction of progesterone receptor in MCF-7 cells occurred on all ECM proteins, but was significantly lower on laminin. These experiments were all performed in the presence of charcoal stripped FBS.

Next, serum-free culture conditions were developed that would permit estrogen-responsiveness to determine the direct effects of isolated ECM proteins, as serum contains hormones, growth factors and ECM proteins. When plated and cultured in serum-free media without growth factors, estrogen did not stimulate proliferation of either cell line. Insulin and growth factors (EGF and IGF-I) have been reported to permit various estrogen-mediated responses in MCF-7 cells and T47D cells, when used at submaximal concentrations. Thus, EGF and IGF-I dose response curves were determined on collagen I for both MCF-7 and T47D cells. EGF and IGF-I significantly stimulated proliferation at concentrations as low as 5 and 25 ng/ml for both cell lines. At these concentrations of growth factors, estrogen stimulated proliferation of MCF-7 cells on collagen I, and T47D cells on collagen I, fibronectin and vitronectin (NOTE: we have repeated some of the serum-free experiments since the first annual report, which was necessary to obtain statistical differences, and determine statistical significance). However, following 4 d of treatment, DNA content of estrogen treated cells was only 20-30% greater than control treated cells on stimulatory ECM proteins for both cell lines.

These serum-free conditions were also used to determine if ECM proteins altered estrogen-induced progesterone receptor in MCF-7 cells. Similar to serum-containing media, E-induced progesterone receptor occurred on all ECM proteins, except laminin. In contrast to proliferation experiments in the absence of serum, we found that estrogen stimulated progesterone receptor

expression to the same extent in the absence of serum as in the presence of serum, when IGF-I and EGF were present. This suggests that these two estrogen-mediated events are dissociated. Our serum-free proliferation assay was insensitive and unlikely to be useful in determining the mechanisms involved in altered estrogen responsiveness on different ECM proteins.

In the next grant period, we first needed to develop a more sensitive assay to measure estrogen-induced proliferation in serum-free culture. We used thymidine incorporation and cell cycle staging to determine how estrogen regulates proliferation of the breast cancer cell lines, MCF-7 and T47D and the ER negative MDA MB231 cells on different ECM proteins. Next, we modified the thymidine assays for loosely attached cells, common in cancer cells plated in serum-free media. Cell cycle analysis revealed that a high percentage of MCF-7 cells were in S-phase even in the absence of serum, estrogen and growth factors, and that a pure anti-estrogen (ICI 182,780) blocked proliferation. Pretreatment with ICI 182,780 reduced basal proliferation and greatly enhanced the estrogen-induced thymidine incorporation. The increased sensitivity made it possible to further examine the mechanisms involved in altered estrogen responsiveness on different ECM proteins.

We report that estrogen-induced proliferation and progesterone receptor induction in two ER positive breast cancer cell lines, MCF-7 and T47D, occurs on collagen I (Col I), Col IV, FN and vitronectin (VN). However, estrogen-induced proliferation and PR induction were significantly lower when cells were cultured on LM. Altered estrogen responsiveness of MCF-7 and T47D cells was not due to cellular ER concentrations. Furthermore, culture of cells on LM did not prevent all mitogenic responses, as IGF-I or EGF induced proliferation on LM. Instead, culture

of cells on LM inhibited estrogen-stimulation of estrogen response element (ERE) reporter activity, thereby inhibiting the transcriptional activity of the ER.

In the final grant period we have examined the effect of LM to alter another ER reporter element: the AP-1 promoter element, with or without co-transfection with ER to amplify ER expression. We have also analyzed whether LM inhibits ERE-mediated transcription by reducing the affinity of E for ER or by reducing E efficacy. Next, as per S.A. #2 and #3 we tested the ability of stromal cells that we have isolated from normal breasts (primarily fibroblasts) and from breast cancer biopsies (primarily myofibroblasts) to alter breast cancer cell proliferation and E-action in breast cancer cells. These results, however, did not result in any consistent changes. Since these results were negative and since we have made the important discovery that LM significantly alters E-responsiveness in breast cancer cells we focused on the effects of LM and its classic receptor, the alpha 6 beta 1 integrin on tumor growth in vitro and in vivo and its alteration of E-responsiveness which may have direct relevance to current breast cancer therapies. This grant has also spawned additionally studies in our lab to examine the developmental and steroidal regulation of ECM proteins and integrins in the normal mammary gland. We have found that ECM protein and integrin expression are altered during normal mammary gland development and are regulated by ovarian steroids. Cumulatively, these studies have demonstrated that 1) ECM proteins may potentiate or block E action in breast cancer cells, 2) specific ECM proteins appear to block antiestrogen treatment of breast cancer tumors in vivo, 3) normal mammary epithelial cell proliferation may be regulated by a complex interactions between growth factors, growth factor receptors, growth factor binding proteins, steroids, steroid receptors, extracellular matrix proteins and integrins. Identification of either acquisition or loss of E-action by specific

ECM proteins, should shape future research in determining how ECM may influence normal mammary growth and tumor growth.

## **BODY**

### **Experimental Methods:**

#### Materials

Culture media, phenol red-free DMEM/Ham's F12, was obtained from Sigma Chemical Co. (St. Louis, MO). Rat Col I, mouse Col IV, ultrapure-entactin-free mouse LM, and human VN were purchased from Becton/Dickinson Labware (Bedford, MA). Human FN was purchased from ICN Biomedicals, Inc. (Aurora, OH). The antiestrogen ICI 182,780 was a gift from ICI Pharmaceuticals (Macclesfield, Cheshire, England). Radioinert R5020 (promogestone), [17 $\beta$ -Methyl-<sup>3</sup>H]Promogestone (R5020, 85 Ci/mmol), and Methyl-<sup>3</sup>H-Estradiol-17 $\beta$  (120 Ci/mmol) were purchased from New England Nuclear Corporation (Boston, MA). Methyl-<sup>3</sup>H-thymidine (50 Ci/mmol) was purchased from ICN Biomedicals, Inc. Nonessential amino acids (NEAA), gentamycin, certified FBS, and insulin were obtained from Life Technologies, Inc. (Grand Island, NY). All culture plates were purchased from Corning Laboratories, Inc. (Corning, NY). Charcoal/Dextran treated DCC-FBS was from Hyclone Laboratories, Inc. (Logan, UT). Insulin-like growth factor-I (IGF-I) was purchased from GroPep Pty. Ltd. (Adelaide, Australia). Poly-L-lysine and all other hormones and growth factors were obtained from Sigma Chemical Co. (St. Louis, MO).

#### Cell lines, culture conditions, plasmids and ECM coating

MCF-7 and T47D human ER positive, estrogen-responsive breast cancer cells were kindly provided by Dr. W. Helferich, Univ. Illinois, Urbana, IL and Dr. B.K. Vonderhaar, NIH, Bethesda, MD, respectively. ER negative MDA MB231 cells were also provided by Dr. W. Helferich. MCF-10A TG1 and MCF-10A TG3c cells were provided by Dr. M. Shekhar,

Karmonos Cancer Inst., Detroit Mich. These lines were derived from MCF-10A spontaneously immortalized normal human breast cells. The T24 ras transfected TG series include the non-transformed TG1 and the transformed TG3c. These cells were cultured in DME/F12 containing 5% FBS, 10 nM estrogen, 125 ng/ml insulin, and 50 ug/ml gentamycin for routine culture. Experimental media contained phenol-red free DME/F12 + 5% DCC and 50 ug/ml gentamycin and was used in all serum-containing experiments. Serum-free experimental media contained phenol-red free DME/F12 with 125 ng/ml insulin, NEAA, gentamycin with or without 5 ng/ml EGF and 25 ng/ml IGF-I, as noted. All cells were incubated in 5% CO<sub>2</sub> at 37 C.

ERE-luciferase (pERE-tk109-luc) and control TK-luciferase (ptk109-luc) were kindly provided by Dr. Barry Gehm, Northwestern University Medical School, Chicago, IL (Gehm et al., 1997). A  $\beta$ -galactosidase plasmid, p6RL, was provided by Dr. Donald Jump, Michigan State University, East Lansing, MI.

Col I, Col IV, LM, FN were coated at 6.25 ug/cm<sup>2</sup>, PLL at 12.5 ug/cm<sup>2</sup> and VN at 500 ng/cm<sup>2</sup> as previously described (Xie and Haslam, 1998).

#### Serum containing DNA assays

MCF-7, T47D or MDA MB 231 cells were plated at  $7.5 \times 10^3$  cells/cm<sup>2</sup> in 24-well dishes for 24 h, switched to experimental media for 2 d, media changed, treatments applied for 4 d and cells harvested for DNA assay. Treatments were changed every 2 d. DNA content was determined by a fluorometric assay using Hoescht 33258 dye (West et al., 1985).

#### Serum-free DNA assays

MCF-7, T47D or MDA MB 231 cells were plated at  $3.75 \times 10^4$  cells/cm<sup>2</sup> in 24-well dishes in serum-free experiment media for 2d with 5 ng/ml EGF and 25 ng/ml IGF-I, and treatments added for 4 d similar to serum containing DNA assays.

#### MCF-7 proliferation on myofibroblast or fibroblast ECM coated coverslips



Breast cancer myofibroblasts or normal breast fibroblasts were isolated by collagenase digestion and differential trypsinization as previously described. Fibroblast were elongated cells that stained positive for vimentin and myofibroblasts had a similar appearance with large cytoskeletal "stress filaments" containing smooth muscle actin. Both cell types were plated at  $5 \times 10^5$  cells/well on PLL (100 ug/ml) coated coverslips ( $2 \text{ cm}^2$ ). Cells were cultured for 4 days in SFM media containing 5 ng/ml EGF and 25 ng/ml IGF-I. Media was changed every 2 d. Cells were removed by successive: rinses with HBSS and incubations in 5 mM EDTA containing HBSS. After cells were removed, MCF-7 cells ( $1 \times 10^5$ ) were plated on fibroblast or myofibroblast ECM coated coverslips or control PLL coverslips. Thymidine assays in the presence or absence of E after ICI treatment were performed as described above.

### $^3\text{H}$ -thymidine assays

Cells were assayed for  $^3\text{H}$ -thymidine incorporation after treatment with the anti-estrogen ICI 182,780 and recovery in estrogen as previously described. Briefly, cells were plated at  $1 \times 10^5$  cells/ $\text{cm}^2$  in 24 well dishes in serum-free experiment media with 5 ng/ml EGF and 25 ng/ml IGF-I for 24 h, then  $2 \times 10^{-7}$  M ICI 182,780 added for 2 d. Cells were subsequently rinsed and treatments ( $2 \times 10^{-7}$  M ICI 182,780, no treatment or  $1 \times 10^{-8}$  M estrogen) added for 20 h. One uCi methyl- $^3\text{H}$ -thymidine/well was added for 2 h and cells transferred to GFC filters (Whatman International, Ltd., London England), the well rinsed 1X with HBSS, and the filter rinsed 2X each with ice cold HBSS, 10% TCA and 90% ethanol. Filters were transferred to liquid scintillation vials and radioactivity determined.  $^3\text{H}$ -thymidine incorporation per well was adjusted by DNA content per well.

### Steroid hormone binding assay

Ligand binding assay were used to determine estrogen receptor and progesterone receptor content as previously described (Xie and Haslam, 1998). Briefly, for ER binding assays, cells were cultured in 24-well plates in serum-free treatment media with EGF (5 ng/ml) and IGF-I (25 ng/ml) for 3 days, incubated with 14 nM  $^3\text{H}$ -estradiol with or without 250-excess unlabeled estradiol (3.5  $\mu\text{M}$ ) for 1 h at 37 C. Cells were transferred to GFC filters and rinsed 3 times with HBSS. Filters were transferred to liquid scintillation vials and radioactivity determined. PR content was determined after 3 d estrogen or control treatment. 8 nM radiolabeled R5020 plus

500-fold excess dexamethosone (total binding) or 500-fold excess unlabeled R5020 (non-specific) were added for 1 h at 37 C. Dexamethosone was added to suppress R5020 binding to glucocorticoid binding sites. ER and PR per well were adjusted by DNA content per well.

#### ERE luciferase, AP1 CAT and luciferase, protein and $\beta$ -galactosidase assays

Cells were plated at  $1.2 \times 10^5/\text{cm}^2$  in 6-well dishes in serum-free treatment media with EGF (5 ng/ml) and IGF-I (25 ng/ml) for 24 h, transfected with 2 ug/well ERE-luciferase or TK-luciferase or 1.5 ug/well p6RL ( $\beta$ -galactosidase plasmid) using Superfect transfection reagent (Qiagen, Inc., Valencia, CA) for 2 hours. Treatments, estrogen (10 nM), no treatment, or ICI 182,780 (200 nM), were added directly following transfection for 24 h, and cells lysed for luciferase, protein and  $\beta$ -galactosidase assays in luciferase reporter lysis buffer.

Luciferase activity was determined using a Promega Luciferase Assay System with Reporter Lysis Buffer (Promega Corp., Madison, WI), as per Promega Technical Bulletin 161 and read on a Turner TD-20e luminometer (Turner Designs, Inc.).

Protein content was determined by the Bio-Rad Protein microtiter plate assay (Bio-Rad Laboratories, Hercules, CA) and read at 595 nm.

B-galatosidase content was determined using Chlorophenol Red- $\beta$ -D-galactopyranoside sodium salt (CPRG, Boehringer Mannheim, Indianapolis, IN) and read at 575 nM, B-galactosidase was used as a standard.

(AP1)4-TK-CAT was a generous gift of Dr. Dany Chalbos (INSERM U148, University of Montpellier, Montpellier, France) and were used as described (Philips et al., 1998). CAT enzyme assays were performed on whole cell extracts after normalization for  $\beta$ -galactosidase activity as described (Sambrook et al., 1989). An (AP1)2-PRL-luc was a generous gift of Dr. Michelle Fluck (Michigan State University, East Lansing, MI). Luciferase activity was determined as described above.

### ER Westerns

MCF-7 cells ( $1 \times 10^6$ /100 mm dish) were cultured on LM or Col I with or without added estrogen (10 nM) for 2 d. Cells were scraped into RIPA buffer containing protease inhibitors as previously described (Woodward et al., 1996) and proteins separated by polyacrylamide gel electrophoresis, electrotransferred to nitrocellulose and probed with Mab-17 ER antibody (Gift of R. Miksicek, Michigan State University, East Lansing, MI).

### MCF-7 tumor development in nude mice

Three-four week old female athymic nude mice were acclimatized for 1 week after shipping. MCF-7 cells with no ECM or with Col I (200  $\mu$ g/injection) or LM (50  $\mu$ g/injection) or Col I (200  $\mu$ g/injection) + LM (50  $\mu$ g/injection) were injected in 100  $\mu$ l HBSS subcu in 4 well separated areas in the back of athymic nude mice, 2 cranial and 2 caudal as previously described (Hsieh et al., 1998). At the time of implantation a slow release (3 wk release time) E (1mg/pellet) beeswax (40mg/pellet) pellet or control beeswax pellet was implanted subcu cranial from the 4 cell implants. E pellets were changed every 3 weeks and tumor size and incidence at all 4 injection sites was measured weekly. In some experiments 10 mice/treatment group were split into 2 groups after tumor development for 3 wk in the presence of E: one group the E pellet was replaced with a fresh E pellet and the other group the E pellet was replaced with a control pellet and the mice were injected once a week i.p. with ICI 182780 (5mg/mouse) as previously described (Osborne et al., 1995).

### Alpha 6 integrin transfection and selection

MCF-7 cells were transfected with an alpha 6A or alpha 6B expressing plasmid pRc/CMV  $\alpha$ 6A or  $\alpha$ 6B (generous gift of Dr. A. Mercurio, Harvard Medical School, Boston, MA) with a Neo resistance coding region using Superfect liposome transfection reagent as described above (Shaw et al., 1993). Following transfection, cells were cultured for 3 weeks in the presence of 1 mg/ml G418 sulfate selection media. At this concentration, G418 sulfate kills 100% of nontransfected MCF-7 cells in 2-4 d. After selection, alpha 6 integrin positive cells were maintained in 200  $\mu$ g/ml G418 sulfate (which is also 100% cytotoxic to MCF-7 cells). In addition to selection by

G418 sulfate surviving colonies, cells were grown on LM for 3 passages, with vigorous rinsing. Clones were analyzed for increased alpha 6 expression by immunocytochemistry and attachment to LM and will be currently being analyzed by Western blotting. MCF-7 and MCF-7 alpha 6A and 6B cells were plated for 24 h in SFM on Col I or LM and morphology, number of cells in clumps, number of free cells and number of clumps were measured at this time.

### Statistical analysis

All data were expressed as the mean  $\pm$  SEM. Differences between means were tested for statistical significance using the Student t-test or paired Student t-test where appropriate.

### **Results:**

#### **Estrogen-induced proliferation on ECM proteins in the presence of serum**

Two estrogen receptor (ER) positive, MCF-7 and T47D, and one ER negative cell lines, MDA MB231, were used to study the effects of ECM proteins on estrogen-responsiveness. In the presence of 5% charcoal stripped (DCC) fetal calf serum, attachment of all cell lines was greater than 90% on all ECM proteins (data not shown). In the absence of estrogen, DNA/well of MCF-7, T47D or MDA MB231 cells was similar on plastic, Col I, Col IV, FN and VN, but DNA/well was lower when cultured on LM for 4 d, and significantly lower for both T47D (except plastic) and MDA MB231 cells ( $P < .05$ ) (Figure 1A,B,C).

Estrogen treatment for 4 d significantly ( $P < .05$ ) increased DNA/well of MCF-7 and T47D cells by 1.6 to 1.9-fold on Col I, Col IV, FN or VN (estrogen treatment did not significantly increase DNA content of MCF-7 cells plated on VN,  $P < .075$ ) (Figure 1A,B). MCF-7 and T47D cells plated on LM, were not significantly growth stimulated by estrogen (Figure 1A,B). MDA MB231 cells did not show a proliferative response to estrogen on any ECM (Figure 1C). These

results demonstrate that in the presence of serum, estrogen-induced growth of ER positive breast cancer cells is altered by ECM proteins. However, since serum contains ECM proteins and other attachment factors, such as FN and VN, we wished to investigate the distinct effects of specific ECM proteins, under hormone and growth factor defined conditions. To do this, we developed the minimal serum-free culture conditions that support estrogen responsiveness of the MCF-7 and T47D cells.

### **Estrogen-induced proliferation on ECM proteins in the absence of serum**

Others have found substantially reduced responses to estrogen in the absence of IGF-I and/or EGF in MCF-7 and T47D cells in serum-free media (Page et al., 1983; Van Der Burg et al., 1988; Katzenellenbogen and Norman, 1990). Similarly, we found that in the absence of growth factors, neither T47D nor MCF-7 cells showed a proliferative response to estrogen when plated in serum-free media. Thus, dose response studies measuring DNA content after 4 days culture in growth factor containing media were performed to determine the minimum concentration of IGF-I and/or EGF that were necessary to obtain an estrogen-response when cells were plated and cultured in serum-free media. The minimum concentrations of EGF and IGF-I required for estrogen stimulation of proliferation were 5 ng/ml and 25 ng/ml, respectively on Col I. At these concentrations of growth factors, estrogen increased DNA content of both MCF-7 and T47D cells on Col I (Figure 2 A,B). Next, proliferation of MCF-7 and T47D cells by estrogen in the presence of IGF-I and EGF on all ECM proteins was determined. As before, E stimulated MCF-7 cell proliferation 20-30% on Col I, but not on any other ECM protein (Figure 3A). In T47D cells, estrogen significantly increased proliferation on Col I, FN and VN (Figure 3B). Estrogen did not stimulate proliferation of MDA MB231 cells on any ECM protein (Figure 3C).

These results demonstrate that EGF and IGF-I enhanced estrogen-induced proliferation of MCF-7 and T47D cells on specific ECM proteins, suggesting that growth factor, steroid and ECM pathways converge to mediate cell proliferation.

To determine if LM inhibited all proliferative responses, dose response studies of MCF-7 cells (and are currently being done for T47D cells) on Col I, LM, and FN proteins were performed, using thymidine incorporation. IGF-I stimulated thymidine incorporation at similar concentrations on all ECMs, with a maximal increase of 2.3-3-fold on all ECMs (data not shown). Stimulation of proliferation by EGF also occurred at similar concentrations and induced similar responses on all ECMs. Before addition of growth factors, <sup>3</sup>H-thymidine incorporation was slightly lower than fibronectin and collagen I. However, both EGF and IGF-I were effective at increasing proliferation similarly on all ECMs. These data suggest that culture of MCF-7 cells on LM does not prevent the cells from responding to all mitogens, by leaving the cell cycle and differentiating. This is in agreement with other data that determined that the percentage of MCF-7 cells in S-phase on Col I and LM in the presence of stripped serum was not different.

Cell cycle staging, by propidium iodine staining and flow cytometric analysis, of MCF-7 cells in estrogen-free, serum-free, growth factor-free media revealed that as high as 30% of cells were in S-phase. The anti-estrogen ICI 182,780 significantly reduced the percentage of S-phase cells to 10% (data not shown), indicating that ER may still be activated in these estrogen-starved and serum-starved cells. The activation of ER, by residual estrogen or more likely by growth factor pathways (Ignar-Trowbridge et al., 1996), have masked estrogen-induced proliferation in serum-free media. To increase the sensitivity of our proliferation assays, cells were treated with the

anti-estrogen ICI 182,780 and  $^3\text{H}$ -thymidine incorporation measured after treatment with estrogen as previously described (Weinberg et al., 1997). Thymidine incorporation into DNA was 7-10-fold higher in MCF-7 cells treated with estrogen on Col I or FN than in control treated cells (Figure 4A). Strikingly, MCF-7 cells plated on LM had a significantly smaller 2-fold increase in thymidine incorporation upon estrogen treatment, though this estrogen-induced increase in proliferation was significant.

After ICI 182,780 pretreatment of T47D cells, estrogen increased thymidine incorporation 1.5 to 2-fold on FN or Col I, respectively (Figure 4B). Thymidine incorporation in T47D cells was not stimulated by estrogen on LM. Since T47D cells are only weakly adherent to LM in the absence of serum, we wished to determine whether the strength of attachment to LM contributed to the lack of estrogen-responsiveness. In the presence of serum, the percentage of T47D cells attached to ECM proteins is similar to serum-free culture, but the strength of attachment is greater as evidenced by cell detachment during multiple rinsing steps. We repeated these experiment in serum-containing media. Again, estrogen treatment significantly increased thymidine incorporation on Col I and FN, but not on LM (Figure 4C). Therefore, the strength of adhesion was not a determinant of the lack of estrogen-induced proliferation on LM. MDA MB231 cells treated with ICI, did not exhibit estrogen-inducible proliferation on any ECM protein examined (Figure 4D).

### **Estrogen receptor status**

Since both MCF-7 and T47D cells plated on LM showed little or no proliferation in response to estrogen, we hypothesized that ER levels may be down-regulated when cells were plated on LM.



However, MCF-7 and T47D cells had similar levels of ER regardless of ECM (Figure 5). ER content, as determined by ligand binding, was lower in T47D cells than in MCF-7 cells as has been previously reported (Ruedl et al., 1990). These results indicate that the decrease in estrogen responsiveness of MCF-7 and T47D cells cultured on LM was not due to a decrease in ER concentration or ER binding, since culture of cells on different ECM proteins did not alter radiolabeled estrogen binding to the ER. MDA MB231 cells were ER negative on all ECM proteins.

### **Estrogen receptor variants**

Since several recent reports have suggested that ER splice variants are common expressed in normal cells and cancer cells and could potentially lead to altered E responsiveness, we wanted to determine if culture on laminin induced estrogen receptor splice variants that may have altered estrogen activity. Lysates of MCF-7 cells cultured on LM or Col I for 3 d in the presence or absence of E (10 nM) were probed for ER by Western blot analysis. On LM and Col I, in the presence or absence of E the major band (90%) ran at 67 kDa, consistent with wildtype ER (data not shown). There did not appear to be any differences in the banding pattern or intensity of bands between LM and Col I. On both ECM proteins, however, ER was downregulated by E treatment. Therefore, we did not continue to examine the possibility that altered expression of ER splice variants by LM contributes to altered E action.

### **Induction of estrogen regulated progesterone receptor**

MCF-7 cells contain estrogen inducible progesterone receptors. Since estrogen-induced proliferation was altered in MCF-7 cells it was of interest to determine if estrogen regulation of

PR was altered by ECM. T47D cells were not used in these experiments since they have high levels of PR that are not regulated by estrogen (Horwitz et al., 1982). Estrogen increased PR levels on all ECM proteins except LM (Figure 6). In contrast to estrogen-induced proliferation, estrogen was able to induce PR in the absence of serum to the same extent as in presence of serum (data for serum-containing experiment not shown). Thus, two estrogen-dependent events were down-regulated or lost when MCF-7 cells were cultured on LM.

### **Activation of ERE-luciferase by estrogen on ECM proteins**

The above experiments indicate that estrogen-induced proliferation and PR induction are reduced or absent when MCF-7 cells or T47D cells are cultured on LM. Since lack of response to estrogen on LM was not due to a reduced ER content or binding, we considered the possibility of altered ER function on LM. To test this, we transfected cells with an ERE-luciferase reporter construct, plated cells on different ECMs and analyzed estrogen induction of ERE activity. Loss of activity or significant differences in activity would indicate that ECM proteins might alter cofactors or repressors that permit ER binding to DNA and/or activation of ERE. MCF-7 cells plated on FN and Col I increased ERE-luciferase activity 7 to 10-fold when treated with estrogen, while cells plated on LM had significantly lower induction of ERE-luciferase (Figure 7). As previously reported, T47D cells showed very weak (often non-significant) stimulation of ERE activity, even when co-transfected with estrogen-receptor, preventing accurate testing on various ECM proteins.

### **Dose response ERE-luciferase**

Expression of  $\beta$ -galactosidase from a cytomegalovirus promoter was similar on Col I, FN and LM (data not shown). Additionally, luciferase activity of control tk109-luc plasmid (which does not contain an ERE) transfected MCF-7 cells was not different on different ECM proteins (Fig. 8). This indicates that the transfection efficiency and general transcriptional activity were not influenced by these specific ECM proteins. Thus, LM appears to specifically reduce ERE-mediated transcription. To determine if the reduced activity of E in MCF-7 cells cultured on LM was due to a reduced binding affinity of E to the ER, an E dose response (0.1 nM to 100 nM) experiment for ERE activity was conducted. On all 3 ECM proteins, maximal luciferase activity was reached by 1 nM; on LM and FN luciferase activity highest at 1 nM and Col I at 0.1 nM or 1 nM. These data indicate that cells cultured on LM or FN have the same sensitivity to E and on Col I the cells may have a slightly increased sensitivity. Furthermore, at peak doses, E increased luciferase expression 2.4-fold on LM, compared to control, but increased luciferase expression 7.0 and 11.3-fold on Col I or FN, respectively (Figure 8). Thus, LM reduces E responsiveness by decreasing the efficacy of E in activating ERE mediated gene transcription.

### **Activation of AP1-luciferase or AP-1 CAT by estrogen on ECM proteins**

E action occurs by E binding to nuclear ERs and the E-ER complex binding to activating transcription of genes containing ERE promoter elements. More recently, however, a second ER binding promoter element has been identified, AP-1. The AP-1 fos-jun complex has been known to stimulate proliferation by binding to AP-1 elements in promoters of many genes, including cyclins and cyclin kinases. More recently, an E activated ER has been shown to stimulate transcription of genes by an AP-1 pathway. Since we found that E-induced

proliferation and induction of endogenous E-regulated genes are inhibited when breast cancer cells are cultured on laminin we examined whether E activation of ERE or E activation of AP-1 mediated transcription are altered on laminin when compared to other ECM proteins. We found that E-induced ERE mediated transcription is significantly reduced when cells are cultured on LM. This reduced responsiveness appears to occur by a decrease in the efficacy of E in activating ERE transcription (see above).

We also examined E induction of AP-1 mediated transcription using 2 reporter constructs. The first construct was a generous gift from Dany Chalbos (INSERM Unit E9 148, Montpellier, France) had four AP-1 repeats driving CAT in a pBLCAT8+ plasmid, AP-1-TK-CAT (Phlips et al., 1998, *Molec. Endocr.*, 12:973-985). The second construct was a generous gift from Michelle Fluck (Michigan State University, East Lansing, MI), which contained four AP1 repeats driving a luciferase fragment in a puc18 plasmid. MCF-7 and T47D cells were cotransfected with either AP-1 reporter plasmid and a constitutively active B-gal plasmid and plated with or without 5% DCC FBS on Col I, FN or LM. In all cases transfection efficiency was confirmed by expression of B-gal. We did not find significant AP-1 luciferase or AP-1 CAT activity in either cell type on any ECM protein with or without serum. Since detection of AP-1 activity may require amplification of signal, we also cotransfected MCF-7 cells with ER and either AP-1 reporter plasmid to increase endogenous expression of ER. However, we still did not find significant AP-1 activity. Therefore, these results indicate that 1) E primarily signals through ERE mediated mechanisms in MCF-7 and T47D cell, 2) ERE mediated transcription is significantly inhibited by LM, 3) the efficacy of E stimulation of ERE activity, not the affinity of E, is reduced by LM.

## **Non-transformed human breast cell response to estrogen on ECM proteins**

The MCF-10A TG series has previously been reported to be ER positive and respond to estrogen when plated in 3-D soft agar gels (Dr. M. Shekhar, personal communication). These cells have not been found to proliferate in response to estrogen on tissue culture plastic. Additionally, these cells may have a PR splice variant, as Western blot analysis for PR revealed a strong band below the w.t. molecular weight. Estrogen-responsiveness is rapidly lost in normal mammary epithelial cells in culture. So, this stable cell line series was an excellent opportunity to examine the regulation of estrogen responsiveness in normal cells and as they become transformed. We examined proliferation of both lines in 5% DCC FBS, using DNA and thymidine assays on PLL, Col I, LM and FN. Estrogen did not stimulate thymidine incorporation with short- (18-24 h treatment) or longer (42-48 h) treatment in either the MCF-10A TG1 or TG3c cells on any ECM protein. In the absence of estrogen, thymidine incorporation was lowest for both the TG1 and TG3c cells on LM at 24 h (Figure 9), but by 48 h proliferation was similar on all ECM proteins (Figure 10). DNA content of TG1 and TG3c was not stimulated by estrogen on any ECM protein, either (Figure 11). Initially, we found specific  $^3\text{H}$ -R5020 binding, in the presence of 250-fold excess cold R5020. The binding of R5020 was not altered significantly by either E or culture on different ECM proteins (Figure 12). This implies that there are specific PR, that are not regulated by estrogen or ECM. We compared PR immunocytochemical staining in the TG1 and TG3c to staining in the MCF-7 and T47D cells. MCF-7 cells had very clear staining, while the T47D cells had approximately 10-fold greater staining intensity (this corresponds to the previous results we have gotten with ligand binding experiments and with published results). Alternatively, no staining was present in the TG1 or TG3c cells. A scatchard analysis for PR revealed no PR, only low affinity binding that could not be competed out with unlabeled

progesterone. Therefore, the TG1 and TG3c series, in monolayer culture, do not proliferate in response to estrogen on any ECM and do not have PR, so we did not continue experiments with these cell lines.

### **Proliferation of MCF-7 cells on ECM from normal fibroblasts and breast cancer myofibroblasts.**

Fibroblast and myofibroblast cells were plated on a non-specific attachment factor, PLL, since in the absence of serum, neither fibroblasts nor myofibroblasts attached to tissue culture plastic. Four myofibroblast lines from breast cancer tissue and two fibroblast lines from normal breast were used to determine if stromal derived ECM in normal versus breast cancer alters breast cancer cell proliferation and response to estrogen. Myofibroblasts or fibroblasts were cultured for 4 d in SFM (media changed every 2 d), and removed by EDTA (5 mM) in HBSS and gentle rinsing, leaving ECM proteins on the PLL coated wells (conditioned wells). Confirmation of protein deposition was confirmed by coomassie blue staining of conditioned coverslips. MCF-7 cell proliferation was slightly increased by conditioned coverslips for both myofibroblasts and fibroblasts when compared to unconditioned coverslips. No consistent differences in MCF-7 cell proliferation were noted between myofibroblast and fibroblast conditioned wells (Figure 13).

Estrogen induced thymidine incorporation of MCF-7 cells was not significantly altered by fibroblast or myofibroblast conditioned plates (Figure 13). E-induced proliferation may not be altered because 1) the ECM proteins produced by the different breast stromal cells do not alter E-induced proliferation, 2) myofibroblasts may require active reciprocal interactions with different types of breast cancer cells to alter ECM proteins in an active reciprocal method, 3) different

types of myofibroblasts may exist or myofibroblast ECM production may change with advanced breast cancer, or 4) the breast cancer cells may be responsible for altering their own E-responsiveness by altering ECM themselves. However, we do not have sufficient numbers of stromal cells to further isolate them into different types of the cancer. Likewise, pairing of all the stromal lines with different types of breast cancer cells (variable degrees of differentiation) would incur extensive costs and time and would not be completed in the final 8-12 months of the grant period. Since we have had very positive results that have helped us to better understand loss of E-responsiveness in breast cancer and may lead to different therapeutic strategies we concentrated on extending these studies in the last year of the grant period. In particular we wanted to 1) further examine the mechanism of LM-induced inhibition of E-responsiveness, 2) determine the effect of LM on breast cancer growth and E-responsiveness *in vivo*, and 3) determine if amplification of LM receptors can induce a differentiated state in breast tumors *in vivo*, similar to the normal mammary gland where LM induces functional differentiation.

**Nude mice studies** (studies added because lack of tumor ECM effects (S.A. #2&#3) and because of major finding that LM inhibits E-action *in vitro*)

Our studies indicated that LM has a dominant inhibitory effect on E-action, since in the presence of other ECM proteins LM inhibited E-action. Next, we wanted to determine if LM has a similar effect *in vivo* on breast cancer cell growth and response to E. MCF-7 cells with no ECM or with Col I were implanted subcu in 4 well separated areas in the back of athymic nude mice, 2 cranial and 2 caudal. At the time of implantation (4 wk), a slow release (3 wk) E beeswax pellet or control beeswax pellet was implanted subcu away from the 4 cell implants.



Implantation of MCF-7 cells in Col I slightly increased tumor incidence from 65% to 95%, but substantially increased tumor growth (Fig 14a). Five weeks after implantation, MCF-7 cells implanted with Col I were six to seven times as large (in volume) as MCF-7 cells implanted without Col I (Fig 14b). These experiments demonstrated that MCF-7 cells formed tumors and that ECM proteins increase tumor incidence and tumor growth.

Next we analyzed the effect of LM and Col I on tumor growth and E-responsiveness of tumors. MCF-7 cells were injected subcu with LM, Col I or Col I+LM. All mice received E-treatment for 3 wks (n=10/treatment), at 3 wks half of the mice (n=5/treatment) were switched to the antiestrogen (ICI 182,780), at 6 wks, ICI treated mice were switched back to E. Tumor incidence was slightly higher in Col I (100%) or Col I+LM (94%) containing implants when compared to LM (70%) containing implants (Fig 15a). MCF-7 cells implanted with Col I were 4-5 times larger than LM implanted MCF-7 cells and 2 times larger than Col I+LM implanted cells at 3 weeks after implantation (Fig 15b). Previous results indicate that Col I stimulates tumor growth, these data indicate that LM inhibits Col I induced tumor growth, but does not block it (i.e. Col I+LM tumor size is significantly greater than LM, but significantly less than Col I). ICI treatment for 3 wks reduced tumor volume of cells in Col I by 21% (Fig 15b). When comparing E and ICI treated tumors after 3 wks treatment, E-treated tumors were more than the 3 times the size of ICI treated tumors in Col I. In LM cell volume doubled even in the presence of the antiestrogen ICI 182,780 for 3 wks. In fact, E treated tumors were only 30% greater than ICI treated tumors in LM. Col I+LM containing tumors increased tumor volume slightly in the presence of ICI (15-40%), but increased volume nearly 3-fold in the presence of E. ICI treatment may have long-lasting effects in Col I treated tumors since 3 wks after the end of ICI

treatment, Col I tumors did not significantly increase in size. On the other hand, Col I+LM tumor volume increased more than 50% between 1 wk and 3 wks after ICI removal and tumor volume increased more than 100% in the 3 wks following ICI removal. These results indicate that LM treatment slows tumor growth, but cells become resistant to E and anti-E treatment, becoming relatively E-independent. These results were not predicted from culture experiments since ICI treatment inhibited proliferation in vitro.

Initial gross analysis of tumors indicated that size matched LM tumors had significantly less necrotic tissue than Col I tumors. This may indicate that LM tumors are better vascularized than Col I containing tumors.

**Ongoing Studies** Currently we are analyzing tumor histology, angiogenesis in tumors, ER (to quantitate ER number) and PR (to measure ER function), and presence of ECM proteins and integrins in tumors.

### **Integrin alpha 6 studies** (studies added in Annual report year #2)

MCF-7 cells appear to attach to LM by the "promiscuous" alpha 2 beta 1 integrin, and a LM-specific alpha 6 beta 1 integrin. Strength of attachment to LM, FN, VN and COL IV, as measured by ability of cells to remain attached after multiple rinsing with HBSS, was relatively weak (though nearly all cells do attach to these ECMs), data not shown. Since attachment was weak and since LM significantly decreased E-responsiveness in vitro and in vivo, MCF-7 cells were transfected with alpha 6A and alpha 6B vectors to amplify expression of the alpha 6 integrin subunit. Both alpha 6 A (MCF-7 a6A) and alpha 6 B clones (MCF-7 a6B) attached to

LM in 1-3 h, while w.t. MCF-7 took 8-12 h to attach. We found little difference in attachment or morphologies between MCF-7 a6A and MCF-7 a6B so used MCF-7 a6A in remaining experiments.

In addition to quicker attachment to LM, the MCF-7 a6A cells had striking changes in morphology and binding to other cells. When plated on Col I, MCF-7 cells had slightly more colonies/field and nearly 2 X as many free cells than MCF-7 a6A cells, but colony size (# cells/colony) was not different (Figure 16 a,b,c). When plated on LM, the two cell types did not have different number of colonies/field, but MCF-7 a6A colonies were nearly 4 times as large. On Col I or LM, MCF-7 cells had approximately 40% free cells (cells not in colonies), whereas MCF-7 a6A cells had only 25% free cells on Col I and 5% on LM. These results demonstrate that alpha 6A expression results in 8-fold reduction in free cells and formation of large clumps when cells are plated on LM. This indicates that cell-cell adhesion is increased in MCF-7 a6A cells in addition to cell-ECM adhesion. It is not clear whether increased adhesion occurs as a result of alpha 6A integrin binding between cells or whether other cell adhesion molecules are upregulated. Interestingly, cell-cell adhesion is often lost in cancer cells, especially metastatic cancer cells.

**Ongoing Studies** Ongoing studies are examining how alpha 6 expression affects tumor growth and E-regulated tumor growth. MCF-7 and MCF-7 a6A cells in nude mice and analyze tumor growth, E-regulation and tumor angiogenesis.

## Discussion

We have made several important findings that have answered long standing questions in the breast cancer field in studies conducted under this grant. We have determined that ECM proteins regulate E mediated signaling in breast cancer cells, and may cause ER<sup>+</sup> breast cancer cells to become E-insensitive and even antiestrogen resistant. For more than 20 years researchers have sought to determine what paracrine derived growth factors may permit or block E responsiveness. These studies have determined that growth factors (IGF-I and EGF) are critical for E action, but that ECM proteins may act as master regulators of E-mediated events by regulating gene transcription. These study demonstrates that specific ECM proteins can differentially regulate estrogen-mediated events in breast cancer cells in vitro and in vivo.

We have previously found that normal primary mouse mammary epithelial cells from nulliparous animals proliferate in response to progestin (R5020) on FN and Col IV, but not on PLL, Col I, tenascin, or LM (Xie and Haslam, 1997). We hypothesized that a similar mechanism may exist for estrogen-induced proliferation of breast cancer cells in culture; such that certain ECM proteins may be required for estrogen responsiveness. Instead, MCF-7 and T47D breast cancer cells proliferated on most ECM proteins examined, including Col I, Col IV, FN and VN, in response to estrogen when cells were cultured in the presence of growth factors (IGF-I and EGF) or serum. However, in the presence or absence of serum, breast cancer cells on LM exhibited little to no estrogen-induced proliferation. Furthermore, estrogen did not significantly increase progesterone receptors in MCF-7 cells on LM. Estrogen receptor content was not altered when cells were cultured on different ECM proteins. Additionally, ER splice variants were not

induced or altered by culture on different ECM proteins. However, estrogen was significantly less effective in stimulating ERE activity when MCF-7 cells were cultured on LM, indicating LM specifically inhibits estrogen-mediated transcription of genes containing ERE. E did not induce AP-1 activity on any ECM protein even after amplifying ER, indicating that AP-1 is not a major E-signaling pathway for these cells. When MCF-7 cells were injected into athymic nude mice with LM they grew up to 5-fold slower in response to E than when injected with Col I. Additionally, tumor incidence was slightly lower in LM injected tumors and response to anti-E was substantially reduced. When LM was added to Col I injected cells, LM reduced E-response and anti-E responsiveness. These data support the role of the tumor extracellular microenvironment in influencing hormonal responsiveness and anti-E treatment, critical to tumor growth and breast cancer therapies.

ECM regulated proliferation has not been well studied in the breast. However, LM induction of a differentiated phenotype in cultured normal mammary epithelial cells, including expression of milk proteins and morphological differentiation, have been well studied. ECM proteins have also been clearly demonstrated to alter invasion and haptotaxis of cultured breast cancer cells. ECM proteins regulate cell morphology of breast cancer cells, as culture of MCF-7 cells on LM coated dishes or addition of soluble LM to cells plated on plastic caused cells to form clusters and prevented spreading and migration. Whereas, MCF-7 cells on FN had a flattened morphology and increased cell spreading (Noel et al., 1988; Coopman et al., 1991). Similarly, we have found an increase in rounding and clustering of MCF-7 and T47D cells on LM. Alternatively, LM did not alter the flattened morphology of the ER-negative MDA MB231 cells. Regulation of normal and breast cancer cell proliferation by ECM proteins has also been reported

(Pourreau-Schneider et al., 1984; Elliott et al., 1992; Xie and Haslam, 1997). We have found that normal mammary epithelial cells from nulliparous mice in serum-free culture proliferate in response to progesterone on Col IV or FN, but not on Col I, LM or tenascin (Xie and Haslam, 1997). Using less defined ECM preparations, Pourreau-Schneider and coworkers (1984) examined estrogen responsiveness of MCF-7 breast cancer cells. Little difference in growth responsiveness to estrogen was found when MCF-7 cells were plated on plastic, a crude corneal endothelial cell ECM preparation, or Col I in the presence of serum. This study did find that culture of MCF-7 cells in 3-D spheroid suspension culture enhanced estrogen-induction of PR. This enhanced response to estrogen may have been mediated by secreted ECM proteins, however, since other mammary carcinoma cell lines have been reported to increase secretion of FN when cultured as spheroid colonies in suspension (Saulnier et al., 1996). The present study is the first to demonstrate regulation of estrogen regulated events in breast cancer cells by specific purified extracellular matrix proteins in serum-free conditions.

ECM proteins interact with growth factors to regulate proliferation of many cell types (Elliott et al., 1992; Bohmer et al., 1996; Wary et al., 1996). ECM proteins bind to cell surface integrins and can activate focal adhesion kinase and several intracellular kinases that are also regulated by growth factors, including MAP kinases, protein kinase C, and PI-3 kinase (Bohmer et al., 1996). ECM-integrin binding can enhance, alter or inhibit growth factor mediated responses (Wary et al., 1996). This report and others have demonstrated that IGF-I and EGF may be necessary for normal estrogen action and in some circumstances activate estrogen receptor pathways independent of estrogen. Insulin or IGF-I have been reported to be required for estrogen induction of PR (Katzenellenbogen and Norma, 1990), while Van de Burg and colleagues (1988)

found that MCF-7 cells require insulin or IGF-I to proliferate in response to estrogen. Both studies used serum containing media to plate cells, however. We have determined that MCF-7 and T47D cells plated in serum-free media without growth factors are not growth stimulated by estrogen, but in the presence of IGF-I and EGF, both cells can respond to estrogen on the appropriate ECM protein. Furthermore, IGF-I and EGF increased ERE-luciferase expression 1.5-2-fold in the absence or presence of estrogen (after adjusting for transfection efficiency) in MCF-7 cells on Col I (data not shown). This was not unexpected, as EGF has been demonstrated to activate the unliganded ER through the MAP kinase pathway (Bunone et al., 1996). Also, EGF, TGF- $\alpha$  and IGF-I have been shown to enhance transcription of an ERE-CAT construct independent of estrogen, but this was blocked by the anti-estrogen ICI 164,384 (Ignar-Trowbridge et al., 1996). Again, these cells were plated or cultured in serum-containing media, so it was not clear what effect ECM proteins had on the regulation of estrogen-induced proliferation. These results demonstrate that IGF-I and EGF may activate ER independent of estrogen or synergize with estrogen to stimulate ERE transcriptional activity, but only when cultured on specific ECM proteins. In agreement, Wary and colleagues (1996) have demonstrated that different classes of integrins may either cooperate with mitogens, promoting cellular proliferation, or preventing mitogen action, demonstrating that attachment to an appropriate substrata acts as a master regulator of cell function.

We have determined that culture of breast cancer cells on LM can inhibit estrogen action even if appropriate growth factors are present. However, unlike our studies, many non-transformed cells can completely shift from a differentiation to proliferation responsive state or vice-versa by culture on specific classes of ECM proteins or by ligation of specific integrins. For example, LM



has been reported to induce differentiation or apoptosis and prevent proliferation of many normal cells, while FN or VN block differentiation and induce or potentiate proliferation. Wary and colleagues (1996) found that ligation of FN ( $\alpha 5$ ) or VN ( $\alpha v\beta 3$ ) receptors recruit Shc-Grb2 which activates the MAP kinase pathway and cooperates with mitogens to promote transcription from the Fos-serum response element to permit cell cycle progression. Laminin and the  $\alpha 6$ -LM receptor did not activate this pathway, but instead induced apoptosis. Other researchers have found similar results. For instance, Sastry and colleagues (1996) found that over-expression of alpha 5 (FN receptor) or antisense knockout of alpha 6 (LM receptors) resulted in decreased differentiation of muscle cells and increased proliferation, while differentiation was correlated with enhanced alpha 6 expression and reduced alpha 5 expression. Endothelial cells have been reported to proliferate on FN coated plates, but on a LM-rich extracellular matrix they stop growing and form capillary-like structures (Kubota et al., 1988). We have also found that LM, but not Col I or FN, generally decreased basal cell proliferation in the absence of estrogen of MCF7, T47D and MDA MB231 cells, and significantly decreases estrogen-induced proliferation. However, dose response studies of MCF-7 and T47D cells for IGF-I or EGF on different ECMs revealed that thymidine incorporation occurred at similar growth factor concentrations and at similar rates on different ECM. These data demonstrate that culture of breast cancer cells on LM does not eliminate all mitogenic responses, indicating that inhibition of estrogen mediated events does not occur solely because cells are moved from a proliferative to a differentiation state.

Alternatively, lack of estrogen responsiveness may have occurred on LM because attachment was weaker on LM. Giancotti and Mainiero (1994) have presented a convincing hypothesis that

growth of cells is maximal when moderate adhesion to extracellular matrix proteins occurs, while strong adhesion or lack of adhesion are not permissive to growth. When either T47D cells or MCF-7 cells were plated on LM, the cells appeared less flattened, and were more easily removed during rinsing. Consequently cells cultured on LM may have had a looser adherence to the substratum. Loss of estrogen responsiveness on LM cannot be solely attributed to attachment or strength of attachment, as other ECM proteins that promoted similar attachment under serum-free conditions, such as FN, did not inhibit estrogen responsiveness, and the stronger attachment of T47D cells in the presence of serum to LM did not permit estrogen-mediated proliferation (Figure 4B vs. 4C).

Our data demonstrated that culture of MCF-7 cells on LM interfered with estrogen receptor mediated transcription pathways. Laminin does not directly regulate ER levels though, since breast cancer cells plated on different ECM proteins did not have different ER concentrations, as determined by ligand binding assays. This indicates that ER concentration, and ER binding does not explain altered estrogen function when cells were plated on LM. The decrease in ERE-luciferase activity corresponded well with the decrease in estrogen stimulation of thymidine incorporation in MCF-7 cells pretreated with the anti-estrogen ICI 182,780. Laminin was able to significantly stimulate both proliferation and ERE-luciferase activity, but in both cases the stimulation was significantly lower than cells cultured on FN or Col I. Since PR is an endogenous estrogen regulated protein in MCF-7 cells, we examined regulation of PR induction. Similarly, we found that MCF-7 cells on LM had a small estrogen-induced increase in PR (not significant), while cells on all other ECM proteins had a larger significant estrogen-induction of PR. This indicates that regulation of ER mediated transcription is a major mechanism by which LM

influences estrogen action. We did not examine other estrogen-regulated genes. It is possible that not all estrogen regulated genes are inhibited by LM, as others have shown a dissociation between regulation of different estrogen mediated genes (Huey et al., 1998). Inhibition of steroid coactivators or over-expression of corepressors are probable candidates for the reduced activation of ERE transcriptional activity on LM.

The regulation of estrogen action in breast cancer cells may be particularly relevant to advanced breast cancer. Breast carcinogenesis is frequently accompanied by desmoplasia, pronounced changes in stromal tissue, accounting for as much as 90% of the tumor (Dvorak, 1986). Desmoplastic stroma have altered synthesis and secretion of growth factors and ECM proteins. Misregulated expression and over-expression of ECM proteins including collagens, elastin, FN, LM, tenascin, proteoglycans and glycosaminoglycans have been documented (Ronnov-Jessen et al., 1996). The altered ECM in tumors may increase the metastatic potential of tumor cells and prevent immune responses to the tumor or inhibit tumor cell growth and invasion, depending on the ECM produced (Ronnov-Jessen et al., 1996). Additionally, LM and LM receptors are often expressed by the malignant breast cancers cells as well. Interestingly, these malignant cells are less likely to be responsive to estrogen than non-malignant or normal cells. Our results which have determined that collagens and FN permit, while LM inhibits, estrogen action in breast tumor cells indicate that the altered pattern of ECM expression during progression of breast cancer may alter estrogen responsiveness and growth of the tumor.

We have also determined that ECM produced from stromal cells isolated from normal breast and breast cancer tissue do not consistently alter E-responsiveness of breast cancer cells. It is

possible that 1) different types of stromal cells from either normal or cancer tissue exist that influence growth differently, 2) that the stage or type of cancer may determine how stromal cells regulate ECM production, or 3) that some or all stromal cells drift or senesce in culture.

Alternatively, breast cancer cells themselves are known to produce ECM proteins which may influence their own E-responsiveness. We felt to determine if breast cancer stromal cells alter E-responsiveness of breast cancer cells we would need to have a much higher number of biopsies that would not be economically or temporally possible in this grant period.

Since we made the important discovery that a stromal derived protein, LM, dominantly regulates E-action in breast cancer cells in vitro, we wanted to extend these studies to examine how this ECM protein and its classic integrin may affect breast growth in vivo. We determined, for the first time that Col I supports tumor incidence, E-responsiveness, tumor growth and anti-E responsiveness of injected breast cancer cells. However, LM dramatically slows E-regulated tumor growth in the presence or absence of Col I. A major finding of these studies was that LM tumors not only grow relatively independent of E (albeit slower than Col I injected tumors), but also appear to be anti-estrogen resistant. Furthermore, preliminary analysis of similar sized tumors have revealed that LM tumors appear to have less necrotic tissue, indicating that LM may potentiate angiogenesis in these tumors. These results demonstrate that a stromal derived protein, LM may be responsible for anti-estrogen resistance in breast cancer. This data has critical implications to current secondary breast cancer therapies which commonly include anti-estrogen treatment.

We have recently extended these studies to examine the role of the classic LM receptor,  $\alpha 6\beta 1$ , in breast cancer growth and E-responsiveness. Since the  $\beta 1$  integrin subunit is contained in many integrins ( $\alpha 1\beta 1$ ,  $\alpha 2\beta 1$ ,  $\alpha 3\beta 1$ ,  $\alpha 4\beta 1$ ,  $\alpha 5\beta 1$ ,  $\alpha 6\beta 1$ ,  $\alpha 7\beta 1$ ) and is upregulated with expression of corresponding alpha subunits, we overexpressed the alpha 6 integrin subunit to increase expression of functional  $\alpha 6\beta 1$ . Preliminary analysis of alpha 6 integrin expressing clones have revealed these cells have increased cell-cell clustering, up to 8-fold reduction in number of free cells (cells not in clusters) and increased speed of attachment to LM. We are currently examining general growth characteristics of these cells and their response to E and growth factors. We will soon begin nude mice experiments to determine if expression of this integrin alters tumor growth in vivo.

This study demonstrates for the first time that extracellular matrix proteins influence estrogen action in breast cancer epithelial cells. Since stromal cells are the major source of epithelial basement membrane proteins in the normal gland (Keely et al., 1996) and this pattern is dramatically altered during carcinogenesis, expression of different extracellular matrix proteins may be a major pathway by which stromal cells influence epithelial cell behavior. This study demonstrates that the tumor microenvironment influences epithelial cell responsiveness to hormones and may appear to lead to hormone insensitivity in vivo. The loss of hormone responsiveness in breast cancer is associated with a poor prognosis and substantially limits treatment options. Advancing our understanding of the mechanism(s) by which ECM proteins influence hormone responsiveness and tumor growth may lead to novel therapeutic strategies for the treatment of breast cancer.

## CONCLUSIONS

This study demonstrated that specific ECM proteins can differentially regulate estrogen-mediated events in breast cancer cells in vitro and in vivo. MCF-7 and T47D breast cancer cells proliferated on most ECM proteins examined, including Col I, Col IV, FN and VN, in response to estrogen when cells were cultured in the presence of growth factors (IGF-I and EGF) or serum. Percent cell attachment was near 90% for both cell lines to all ECM proteins. However, in the presence or absence of serum, breast cancer cells on LM exhibited little to no estrogen-induced proliferation. Pretreatment of MCF-7 or T47D cells with the anti-estrogen, ICI 182,780, followed by estrogen treatment confirmed our previous serum-free results, that estrogen is a strong mitogen when cells are plated on collagen I or fibronectin, but little to no estrogen-induced proliferation occurred on laminin. Furthermore, estrogen did not significantly increase progesterone receptors in MCF-7 cells on LM. T47D cells have constitutively high expression of progesterone receptors, that are not estrogen regulatable. Estrogen receptor content was not altered when cells were cultured on different ECM proteins. Furthermore, there was no evidence that expression of ER splice variants were induced or altered by culture on LM. However, estrogen was significantly less effective in stimulating ERE activity when MCF-7 cells were cultured on LM, indicating LM specifically inhibits estrogen-mediated transcription of genes containing ERE. AP1 activity was barely detectable did not appear to be induced by E and was not altered by culture on different ECM proteins. We did not obtain E-regulated proliferation for any normal breast epithelial cells on any ECM, suggesting that these cells have either lost their ability to respond to E or need additional extracellular signals. ECM produced by stromal cells isolated from normal or breast cancer tissue did not significantly change

proliferation of breast cancer cells. However, inconsistencies in results (i.e. increased and decreased proliferation with different stromal cells) suggest that either different tumors have different types of stromal cells or that cells may drift or senesce in culture. Unfortunately these results suggest that to further understand how stromal derived ECM may change during breast cancer and how these changes may influence tumor growth would likely require a very high number of breast biopsies that would take many years and drastically increase costs.

We instead followed up on our very promising data that LM and its cellular receptors may act as master regulators of E action in breast cancer cells. We extended these studies to an in vivo model to determine if LM would still alter tumor growth in a very complex in vivo system. These data found that LM not only reduced breast cancer cell growth and response to E, that tumor cells implanted with LM may actually become resistant to anti-estrogens. This is an effect that we did not observe with LM in vitro. We believe this is the first endogenous protein that has been shown to induce estrogen and anti-estrogen resistance to breast cancer cells. In vitro LM substantially reduced or eliminated E responses, but anti-E treatment always significantly lowered E-action on all ECMs. Additionally, initial analyses of tumors from these nude mice have revealed that LM may increase angiogenesis. Since certain fragments of LM have been reported to support angiogenesis and others inhibit angiogenesis, it would be of interest to determine which LM fragments effect E-action and which effect angiogenesis in this tumor model. These data support the role of the tumor extracellular microenvironment in influencing hormonal responsiveness, critical to tumor growth and breast cancer therapies.

In addition to extending our alpha 6 integrin studies (examining the role of the receptor in altering E-action in breast cancer cells in vivo and in vitro) and continuing our nude mice tumor examinations, we are currently extending our studies to examine the role of ECM-integrin interactions in steroid mediated development of the normal mammary gland. We have already found developmental and steroidal regulated ECM proteins in the basement membrane and integrins in both epithelial and myoepithelial cells. Therefore, both normal mammary gland development and its regulation by ovarian steroids and estrogen regulated breast cancer growth appear to be dependent on appropriate ECM/integrin cues, which when altered could lead to loss of responsiveness to ovarian steroids and potentially to loss of response to therapeutic anti-estrogen treatments.

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## FIGURE LEGENDS

### *Figure 1*

Effect of estrogen on proliferation of breast cancer cells on different ECM proteins. DNA content of estrogen receptor-positive, MCF-7 (A), T47D (B), and negative, MDA MB231 (C), cells following 4 d culture in estrogen containing media in the presence of 5% charcoal stripped fetal bovine serum. White bars=control treatment, black bars=10 nM estrogen. Cells were plated on plastic (Plas), collagen I (Col I), collagen IV (Col IV), laminin (LM), fibronectin (FN), or vitronectin (VN). Each value represents the mean  $\pm$  SEM of 3-8 experiments in Panels A and B. Panel C is data from a representative experiment with error bars of replicates within experiment. \*,  $P < .05$  that estrogen treated groups have a higher DNA content than control groups. \*\*,  $P < .05$  that LM control groups have a lower DNA content than control groups on any other ECM protein.

### *Figure 2*

Effect of IGF-I and EGF on estrogen-mediated proliferation in breast cancer cells in serum-free media on collagen I. DNA content of MCF-7 (A) and T47D (B) cells after 4 d treatment with EGF (5 ng/ml), IGF-I (25 ng/ml) and/or estrogen (10 nM) in serum-free media. Cells were plated on collagen I (Col I). Each value represents the mean  $\pm$  sem from 2 experiments. \*,  $P < .05$  that growth factor treated groups have a higher DNA content than control groups. \*\*,  $P < .05$  that estrogen+IGF-I+EGF group is greater than IGF-I+EGF group.

### *Figure 3*

Effect of estrogen on proliferation of breast cancer cells on different ECM proteins in the absence of serum. DNA content of MCF-7 (A), T47D (B) and MDA MB231 (C) cells following 4 d culture in estrogen containing serum-free media was determined. Serum-free media contained 5 ng/ml EGF and 25 ng/ml IGF-I. White bars=control treatment, black bars=10 nM estrogen. Cells were plated on poly-L-lysine (PLL), collagen I (Col I), collagen IV (Col IV), laminin (LM), fibronectin (FN), or vitronectin (VN). Each value represents the mean  $\pm$  SEM of 3 experiments in panels A and B. Panel C is data from a representative experiment with means and error bars of replicates within experiment. \*,  $P < .05$  that estrogen treated groups have a higher DNA content than control groups.

### *Figure 4*

Estrogen induced  $^3\text{H}$ -thymine incorporation in breast cancer cells pretreated with the antiestrogen ICI 182,780 on different ECM proteins. MCF-7 (A), T47D (B), or MDA MB231 (D) cells were pretreated with 100 nM ICI 182,780 in serum-free media, or T47D cells in 5% charcoal stripped FBS (C), for 48 hours, followed by no treatment (Con) or estrogen (10 nM) treatment for 20 hours. Cells were plated on collagen I (Col I), laminin (LM), or fibronectin (FN). White bars=control, black bars=10 nM estrogen. Cells were subsequently labeled with  $^3\text{H}$ -thymidine for 2 hours and CPM/ug DNA determined. All panels are representative experiments, with error bars of at least 3 replicates/treatment. All experiments have been repeated with similar results. \*,  $P < .05$  that estrogen treated groups are greater than control treated groups.

### Figure 5

ER concentration of MCF-7 and T47D cells on different ECM proteins. Specific  $^3\text{H}$ -estrogen binding in MCF-7 and T47D in serum-free media cultured on collagen I (Col I), laminin (LM), or fibronectin (FN) was determined. Serum-free media contained 5 ng/ml EGF and 25 ng/ml IGF-I. Each value represents the mean  $\pm$  SEM from a representative experiment with at least 3 replicates/treatment. All experiments were repeated with similar results. Estrogen receptor was determined by specific  $^3\text{H}$ -estrogen binding and normalized to DNA content.

### Figure 6

Estrogen regulation of progesterone receptor binding levels in MCF-7 cells on different ECM proteins in serum-free media. Specific  $^3\text{H}$ -R5020 binding in MCF-7 in the absence of serum was determined. Media contained 5 ng/ml EGF and 25 ng/ml IGF-I. White bars=control treatment, Black bars=10 nM estrogen. Cells were plated on poly-L-lysine (PLL), collagen I (Col I), collagen IV (Col IV), laminin (LM), fibronectin (FN), or vitronectin (VN). Each value represents the mean  $\pm$  SEM of 3 separate experiments. Cells were cultured in the presence of estrogen for 3 d. Progesterone receptor (PgR) was determined by specific  $^3\text{H}$ -R5020 binding and normalized to DNA content. \*,  $P < .05$  that estrogen treated groups are greater than control groups.

### Figure 7

Estrogen stimulation of ERE-luciferase in MCF-7 cells on different ECM proteins in serum-free media. MCF-7 cells were cotransfected with ERE-luciferase (or TK-luciferase) and  $\beta$ -galactosidase plasmids. Serum-free media contained 5 ng/ml EGF and 25 ng/ml IGF-I. Cells were plated on collagen I (Col I), laminin (LM) or fibronectin (FN). ERE luciferase activity was measured 24 h after ICI 182,780 (100 nM), control, or estrogen (10 nM) treatment. Luciferase activity was normalized to transfection efficiency as measured by  $\beta$ -galactosidase expression/well and were expressed relative to the values that were obtained with the control TK-luciferase plasmid. Each value represents the means  $\pm$  SEM of 3 separate experiments. \*,  $P < .05$  that estrogen treated groups are greater than control groups. \*\*,  $P < .05$  that laminin group treated with estrogen is greater than control treated group, but less than collagen I or fibronectin groups treated with estrogen.

### Figure 8

Estrogen dose response stimulation of ERE-luciferase in MCF-7 cells on different ECM proteins in serum-free media. Culture and treatment conditions were the same as in figure 7. An E dose response 0, 0.1 nM, 1 nM, 10 nM and 100 nM was tested.

### Figure 9

Effect of estrogen on  $^3\text{H}$ -thymidine incorporation of MCF10A TG1 and MCF10A TG3c cells on ECM proteins in 5% DCC FBS after 24 hour treatment. MCF10A TG1 (A) and TG3c (B) cells were plated in 5% DCC FBS on Poly-L-lysine (PLL), collagen I (Col I), laminin (LM) or fibronectin (FN). Control (C) or 10 nM estrogen (E) were added for 18 h. Cells were

subsequently labeled with  $^3\text{H}$ -thymidine for 6 h and CPM/ug DNA determined. Each value represents the mean of at least 3 replicates.

#### *Figure 10*

Effect of estrogen on  $^3\text{H}$ -thymidine incorporation of MCF10A TG1 and MCF10A TG3c cells on ECM proteins in 5% DCC FBS after 48 hour treatment. MCF10A TG1 (A) and TG3c (B) cells were plated in 5% DCC FBS on Poly-L-lysine (PLL), collagen I (Col I), laminin (LM) or fibronectin (FN). Control (C) or 10 nM estrogen (E) were added for 42 h. Cells were subsequently labeled with  $^3\text{H}$ -thymidine for 6 h and CPM/ug DNA determined. Each value represents the mean of at least 3 replicates.

#### *Figure 11*

Effect of estrogen on proliferation of MCF10A TG1 and MCF10A TG3c cells on ECM proteins in 5% DCC FBS. MCF10A TG1 (A) and TG3c (B) cells were plated in 5% DCC FBS on Poly-L-lysine (PLL), collagen I (Col I), laminin (LM) or fibronectin (FN). Control (C) or 10 nM estrogen (E) were added for 4 d and DNA content determined. Each value represents the mean of at least 3 replicates.

#### *Figure 12*

Effect of estrogen on progesterone receptor binding levels in MCF10A TG1 and MCF10A TG3c cells on ECM proteins in 5% DCC FBS. MCF10A TG1 (A) and TG3c (B) cells were plated in 5% DCC FBS on Poly-L-lysine (PLL), collagen I (Col I), laminin (LM) or fibronectin (FN). Specific  $^3\text{H}$ -R5020 binding in MCF10A TG series cells was determined. Cells were cultured in the presence of estrogen for 3 d. Progesterone receptor (PgR) was determined by specific  $^3\text{H}$ -R5020 binding and normalized to DNA content.

#### *Figure 13*

Effect of ECM from breast cancer myofibroblasts and normal breast fibroblasts on proliferation of MCF-7 cells with or without E. MCF-7 cells were plated on PLL coverslips or fibroblast or myofibroblast derived ECM coated coverslips as described in Materials and Methods. MCF-7 cells were pretreated with 100 nM ICI 182,780 in serum-free media, or T47D cells in 5% charcoal stripped FBS, for 48 hours, followed by control or estrogen (10 nM) treatment for 20 hours. Mean and standard error bars are from calculated from results from 6 different cell lines 4 myofibroblast and 2 fibroblast lines. Data are expressed as MCF-7 thymidine incorporation on stromal ECM coated coverslips / MCF-7 thymidine incorporation on PLL coated coverslip \*100, i.e. the percentage of no ECM.

#### *Figure 14*

MCF-7 tumor incidence and tumor size in nude mice in response to Col I. MCF-7 cells were injected subcu into 4-5 wk nude mice in HBSS or HBSS with 200 ug/injection Col I. Four injections of MCF-7 cells were made in each mice (4 potential tumors). At injection, mice were implanted with slow release E (1 mg) beeswax pellets which were changed every 3 weeks.

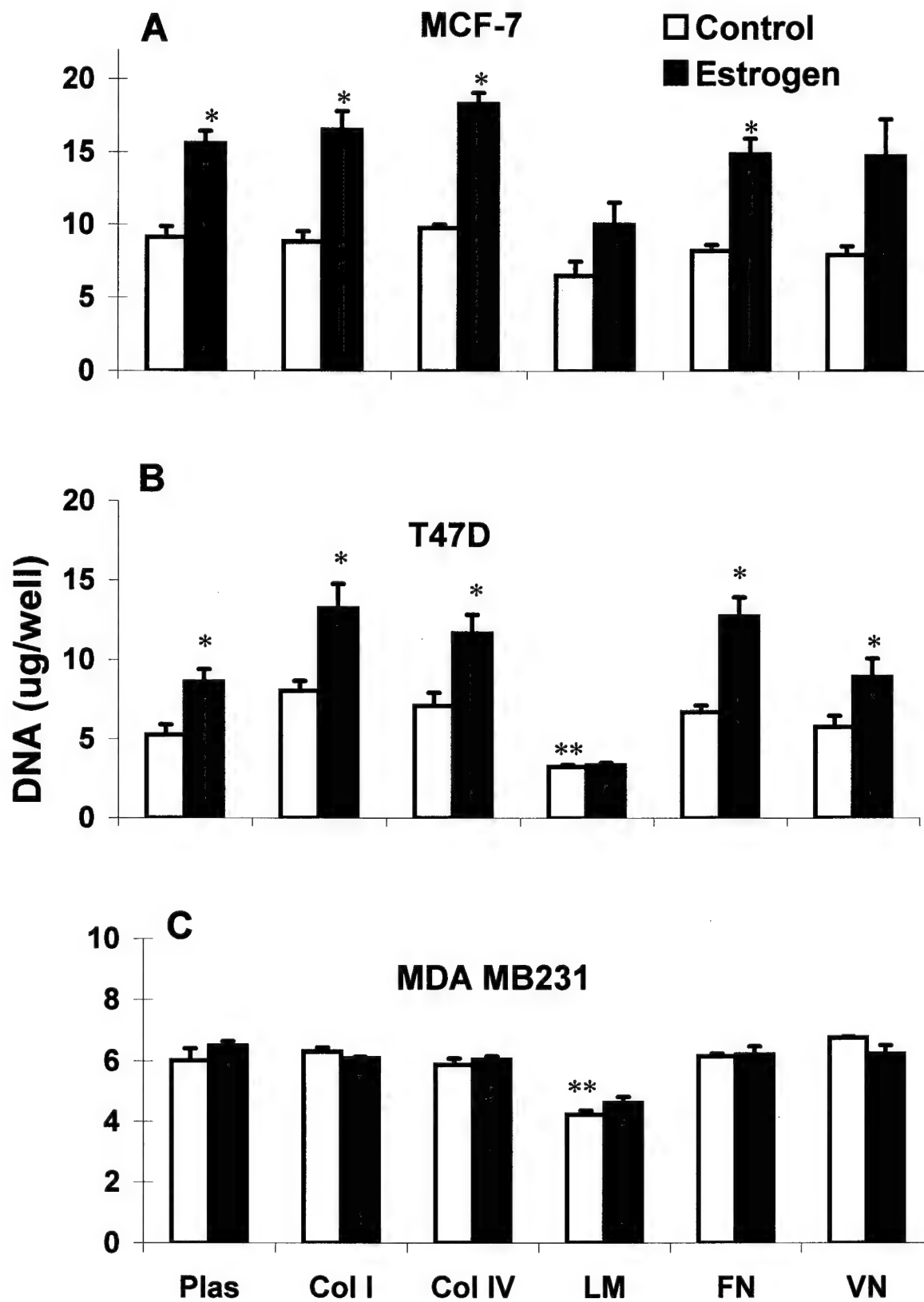
Tumor incidence (A) and size (B) were measured weekly. There were five mice per treatment group.

*Figure 15*

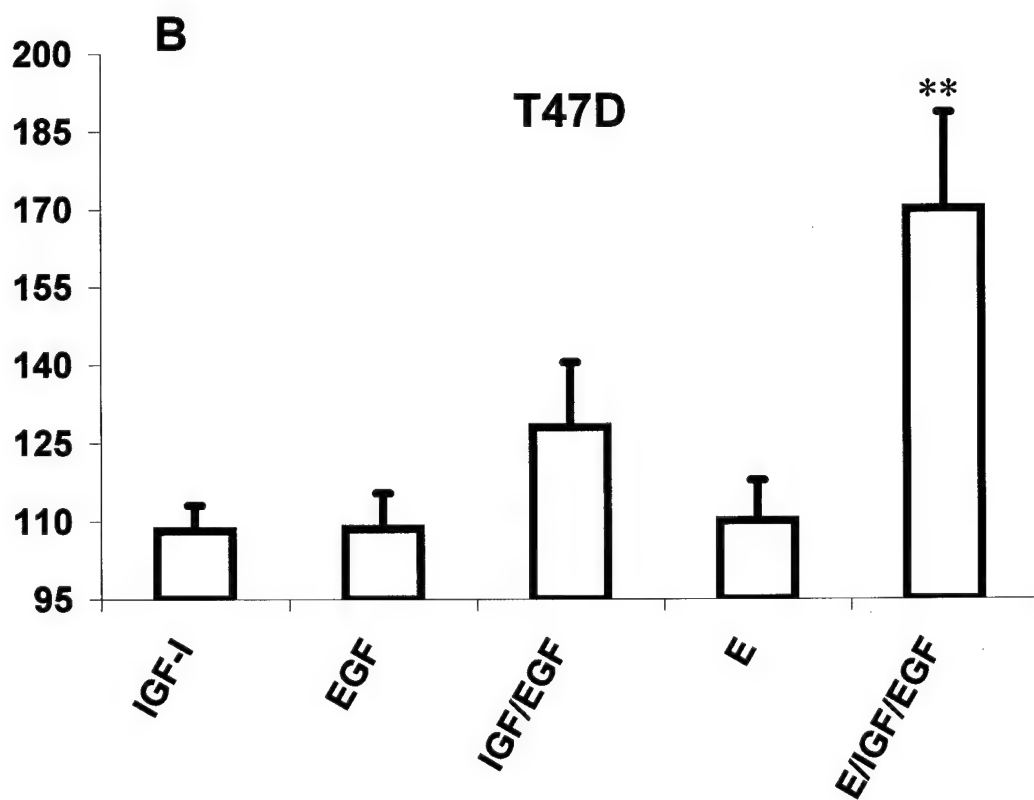
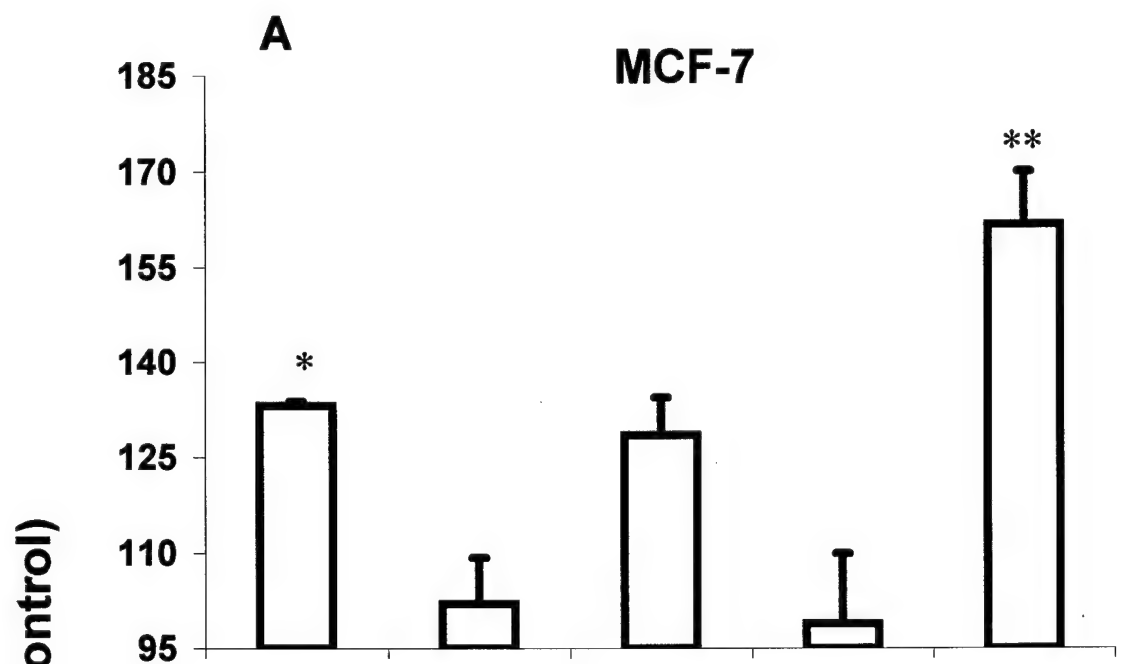
MCF-7 tumor incidence and tumor size in nude mice in response to E or antiestrogen coimplanted with LM, Col I or Col I+LM. MCF-7 cells were injected subcu into 4-5 wk nude mice in HBSS or HBSS with 200 ug/injection Col I, 50 ug/injection LM, or 50 ug/injection LM+200 ug/injection Col I. Four injections of MCF-7 cells were made in each mice (4 potential tumors). At injection, mice were implanted with slow release E (1 mg) beeswax pellets which were changed every 3 weeks (10 mice/treatment). After 3 weeks, E pellets were either replaced or removed and control beeswax pellets implanted. The anti-estrogen ICI 182,780 was injected i.p. weekly in control pellet containing animals between 3 and 6 wks after tumor cell injection (5 mice/treatment). To determine if the anti-estrogen effects were transient or permanent animals treated with ICI for 3 wks were implanted with E following ICI treatment (5 mice/treatment). Tumor incidence (A) and size (B) were measured weekly. Green Lines = Col I, Blue Lines = Col I+LM, Red Lines = LM, solid lines = Estrogen treatment, dotted lines = ICI treatment. Points on line represent mean and standard error of 5 mice/treatment. C) Photograph of 2 nude mice (left mouse tumors were coinjected with Col I, right mouse tumors were coinjected with LM) at end of 6 week estrogen treatment.

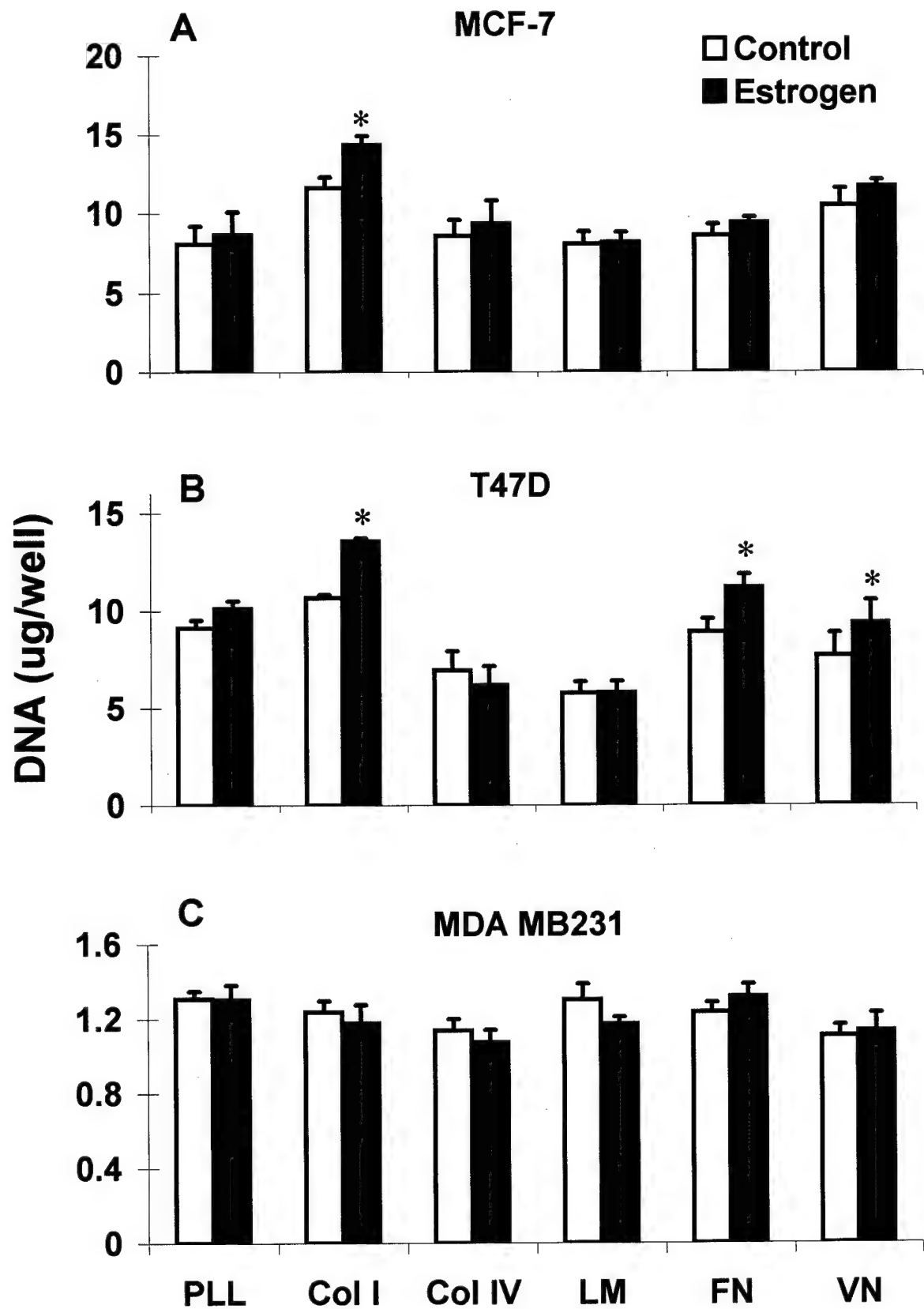
*Figure 16*

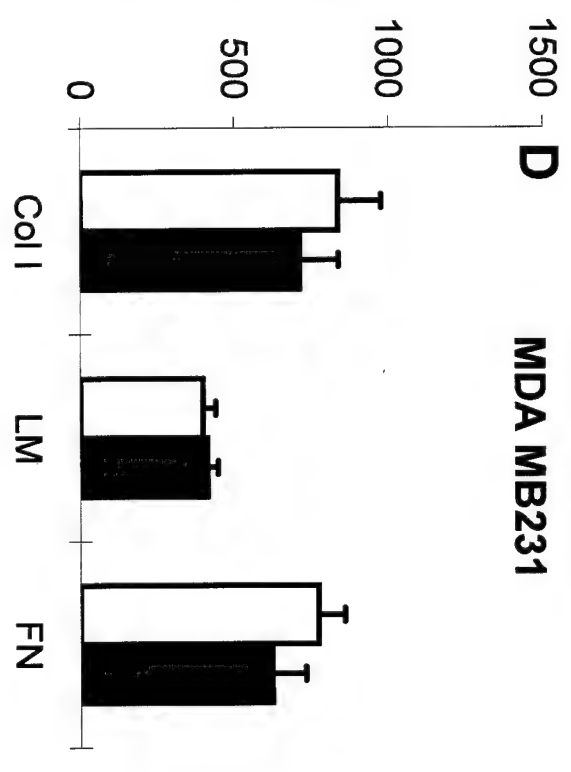
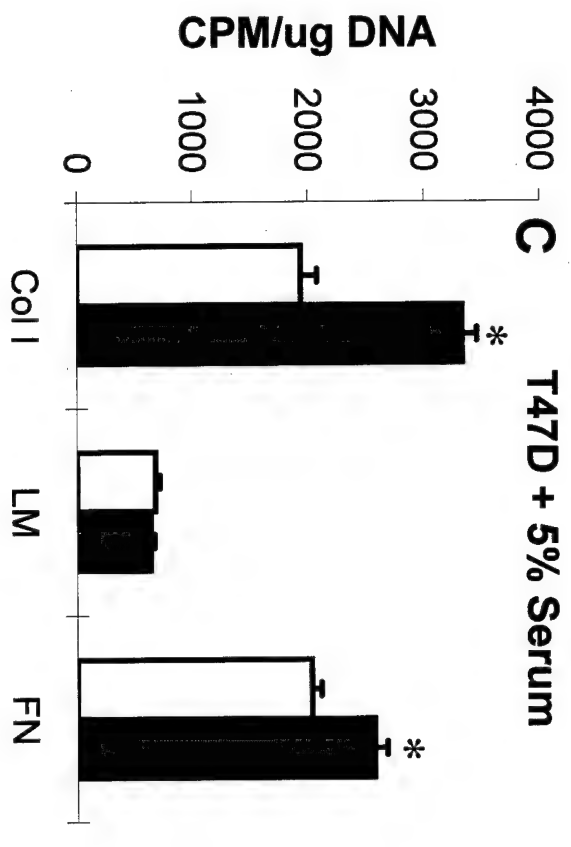
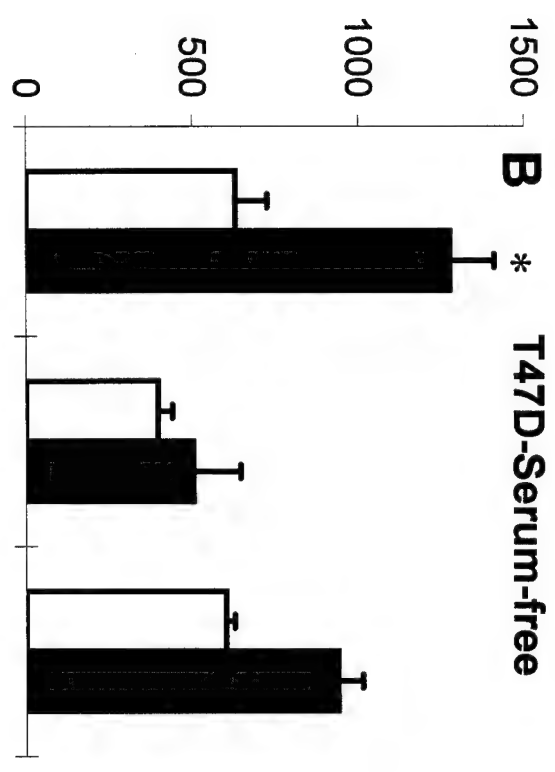
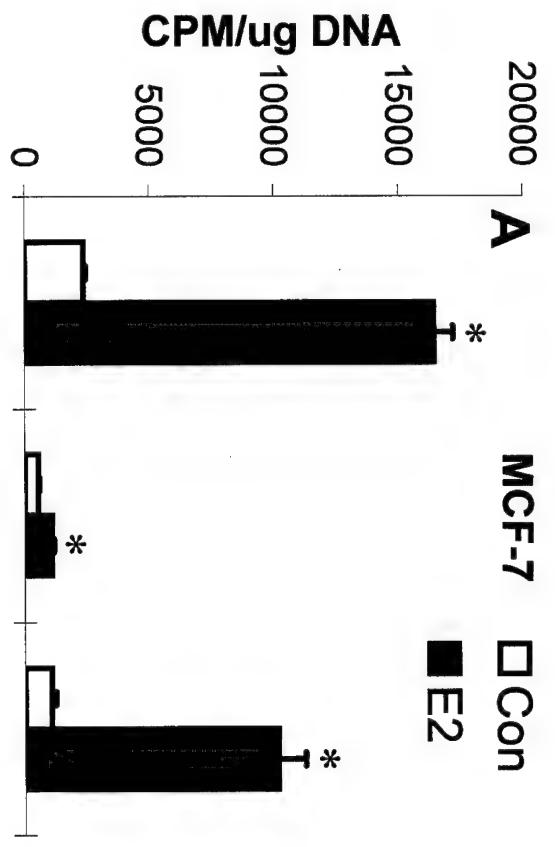
MCF-7 or MCF-7 alpha 6 A expressing integrin subunit cells (MCF-7  $\alpha 6A$ ) plating as free cells or in colonies. MCF-7 or MCF-7  $\alpha 6A$  cells were plated on Col I or LM for 24 h and the number of colonies (A) per microscopic field (200 X total magnification), number of free cells (B) per microscopic field and number of cells per colony (colony size) (C) were counted. Means and standard errors were calculated from a minimum of six separate areas counted for each cell type on each ECM protein.

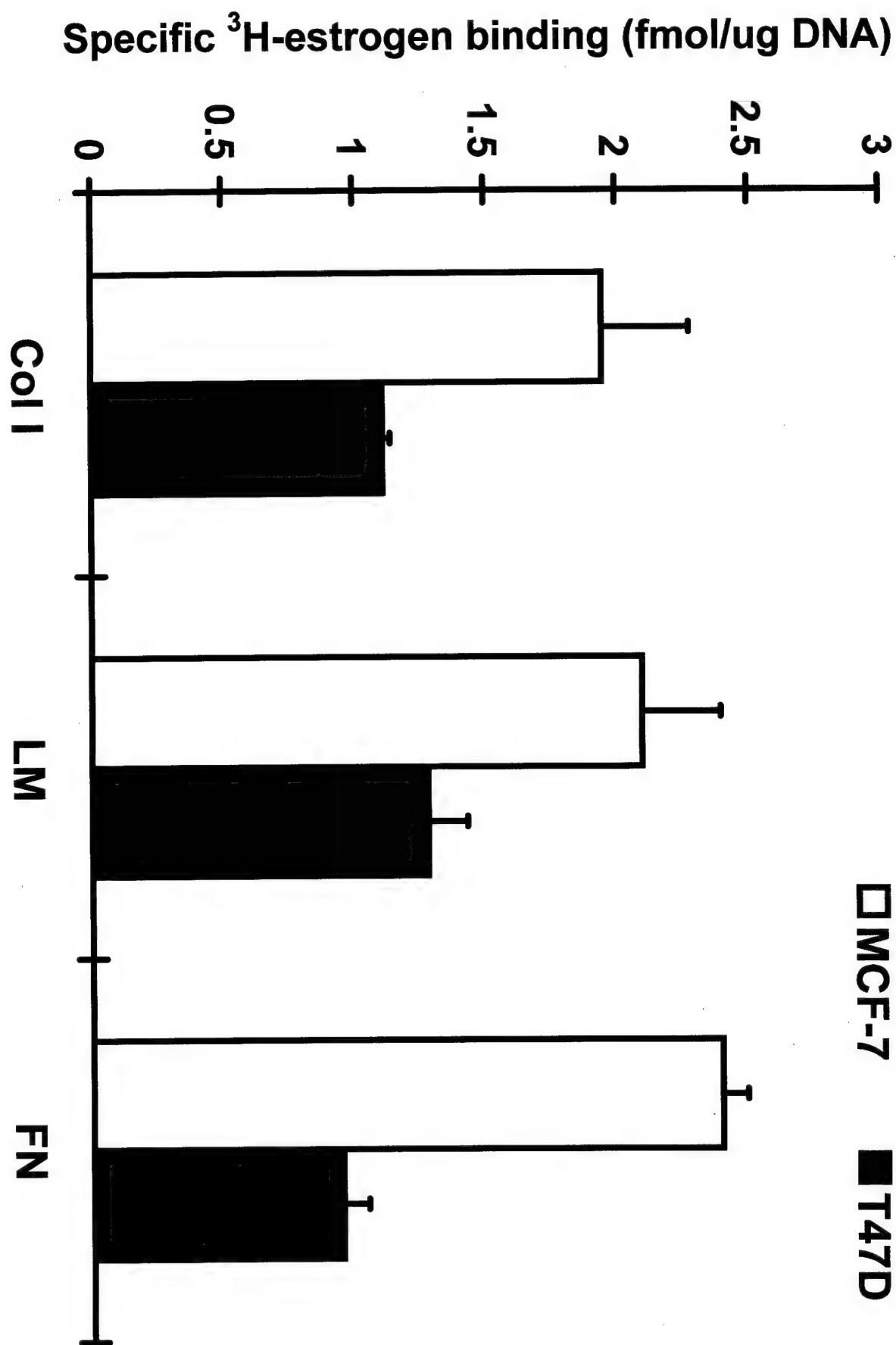


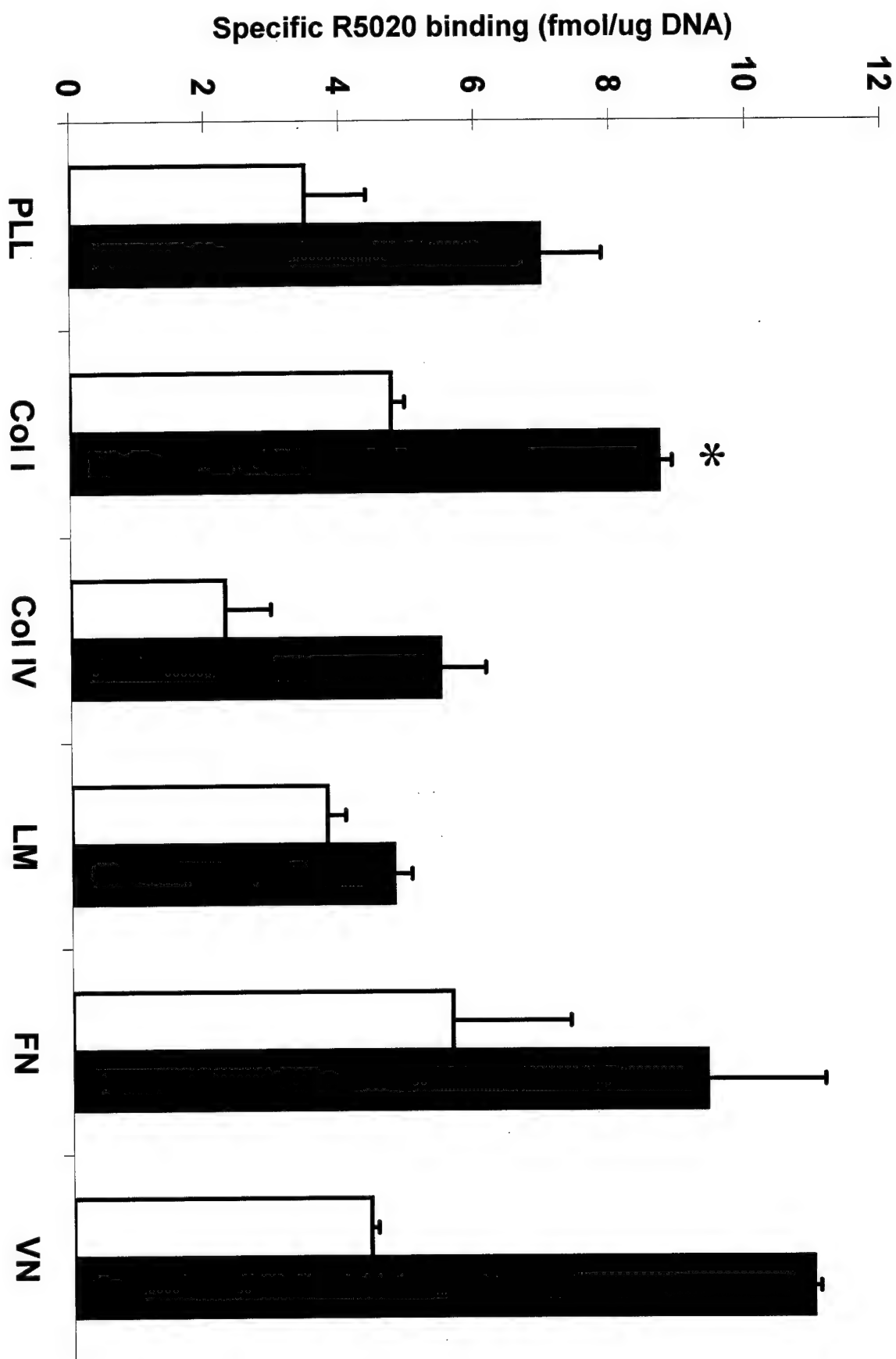


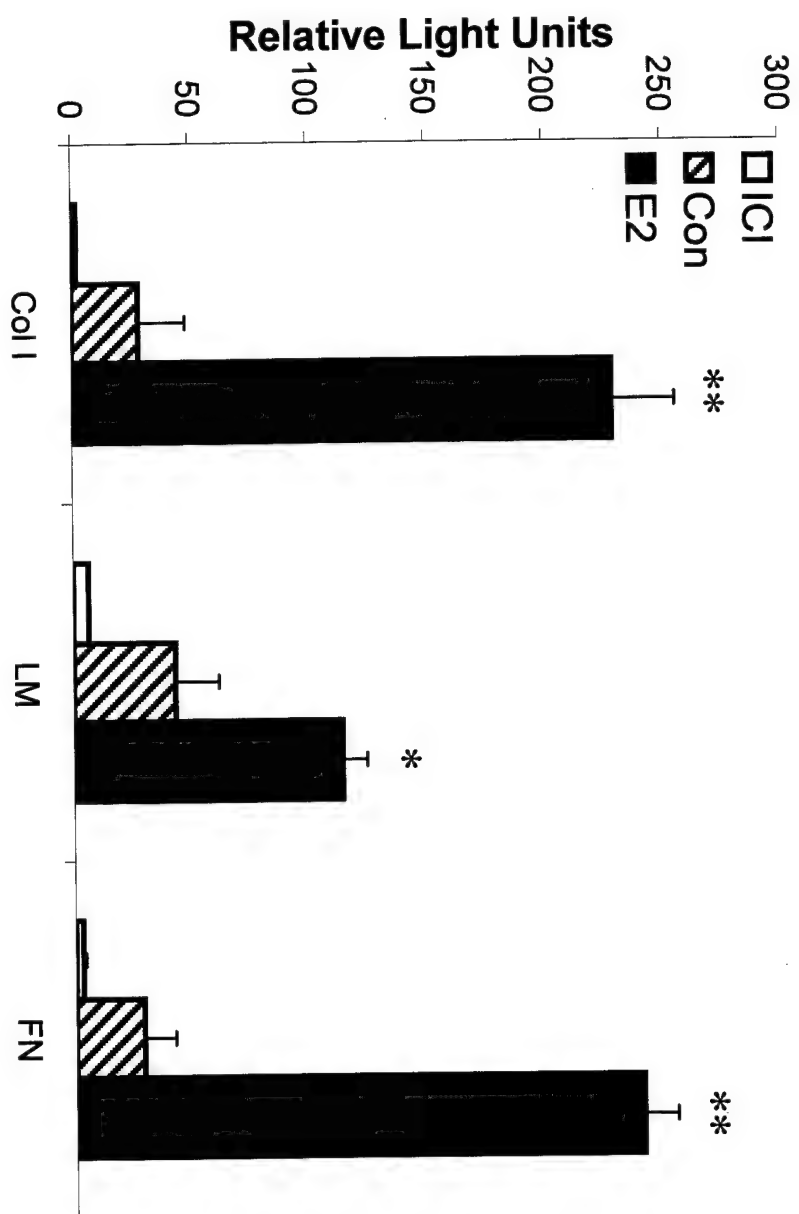


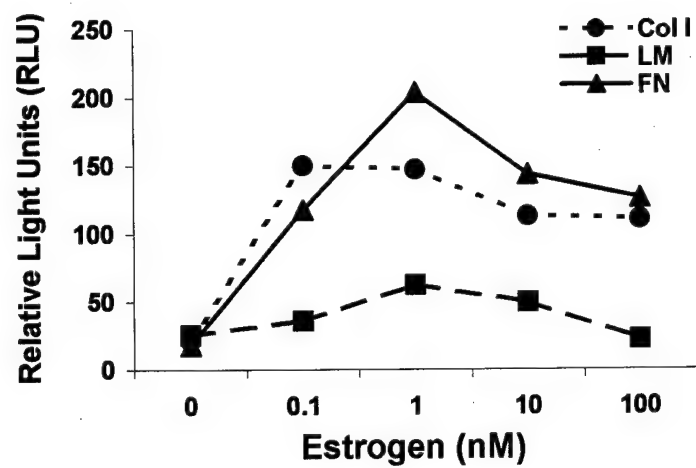


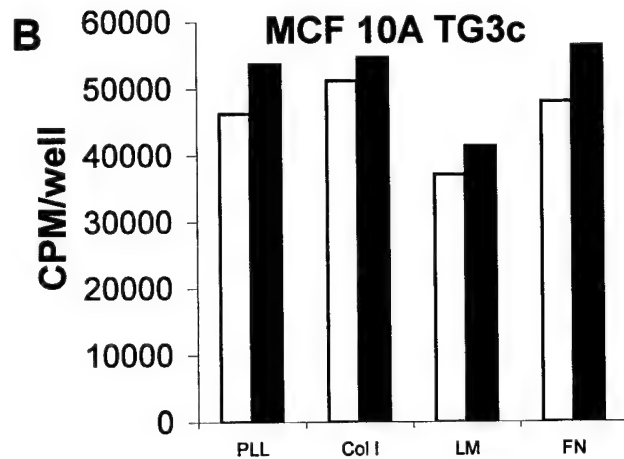
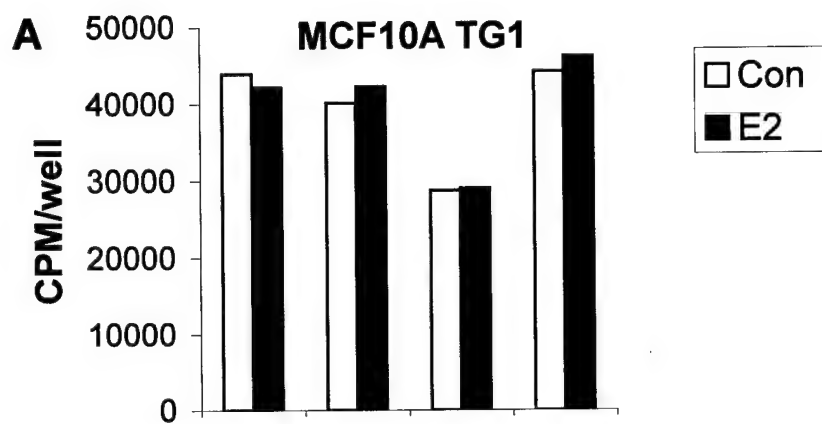




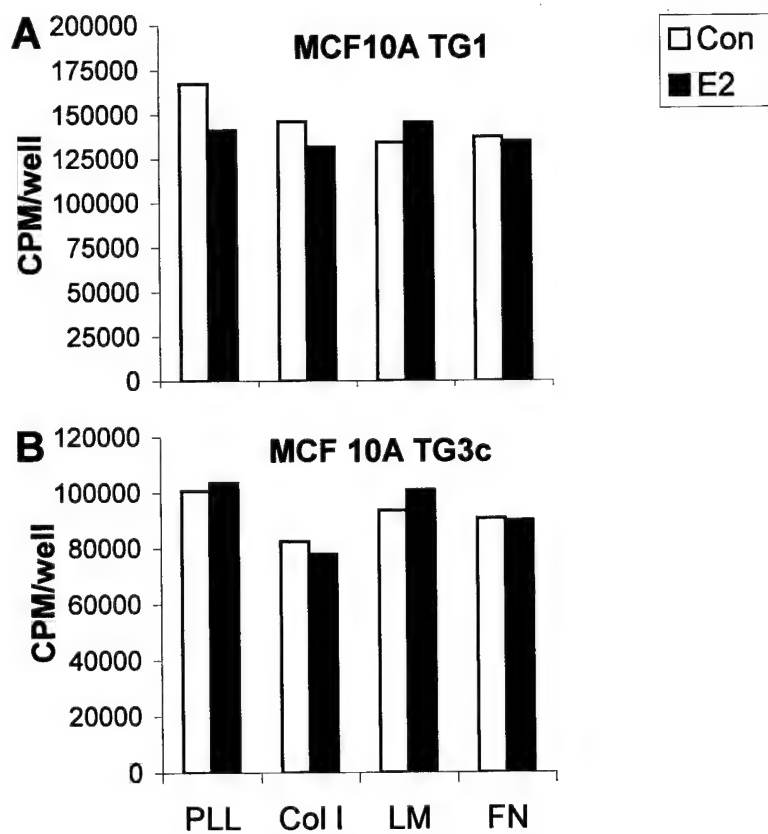


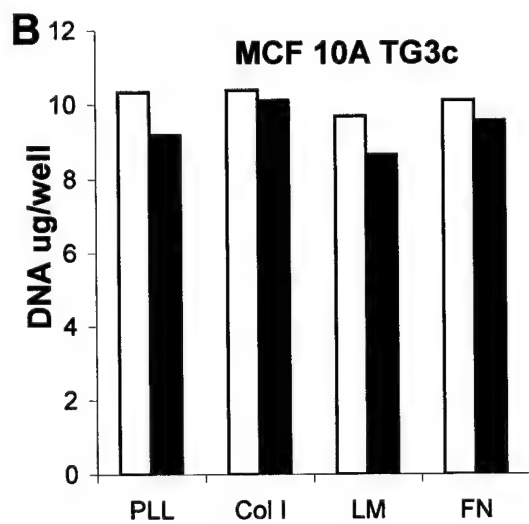
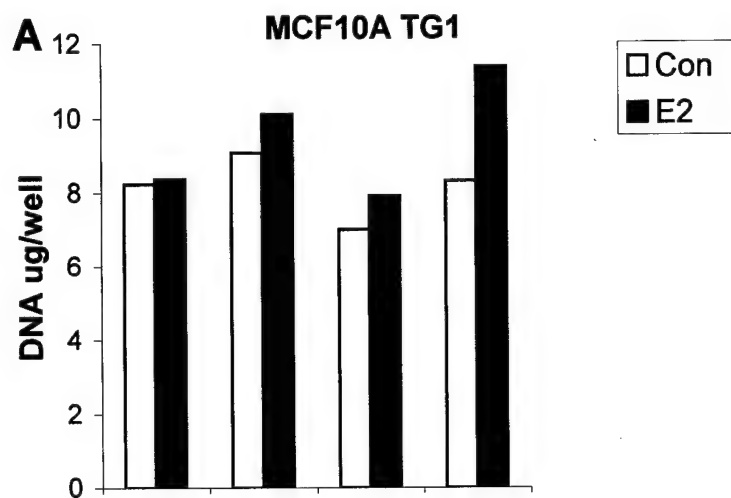


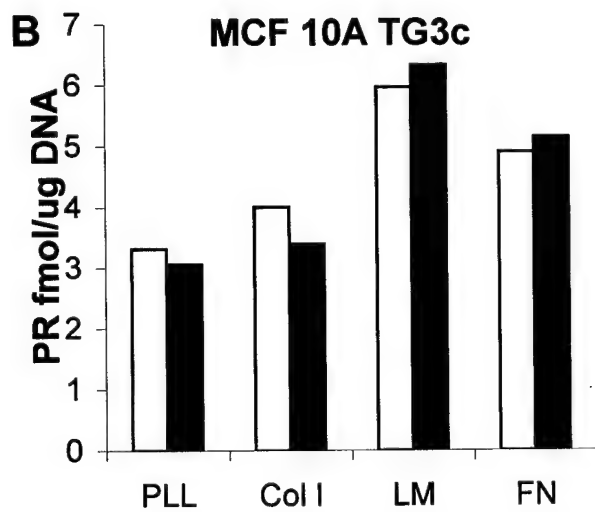
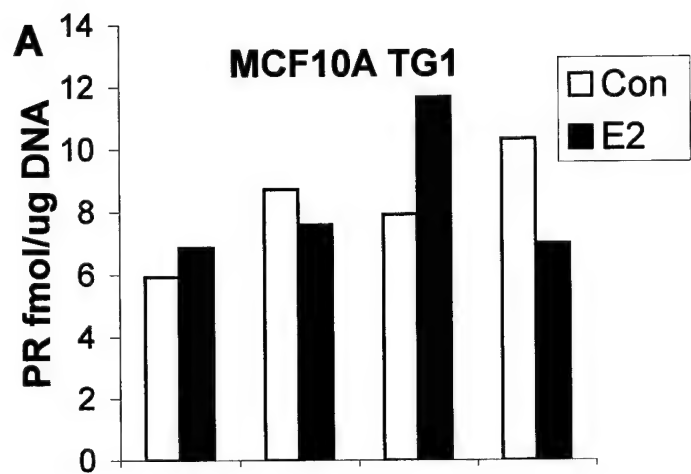


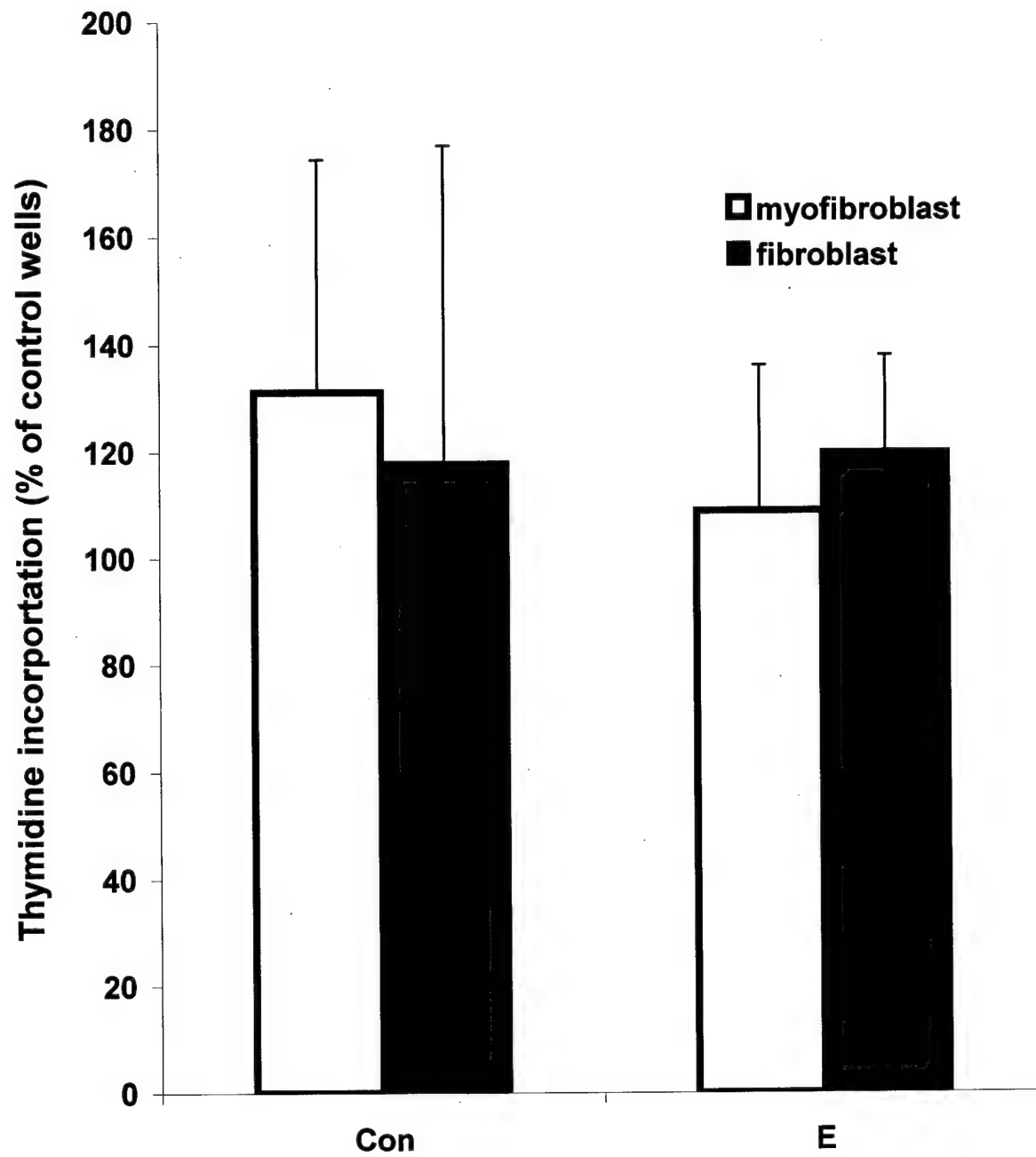


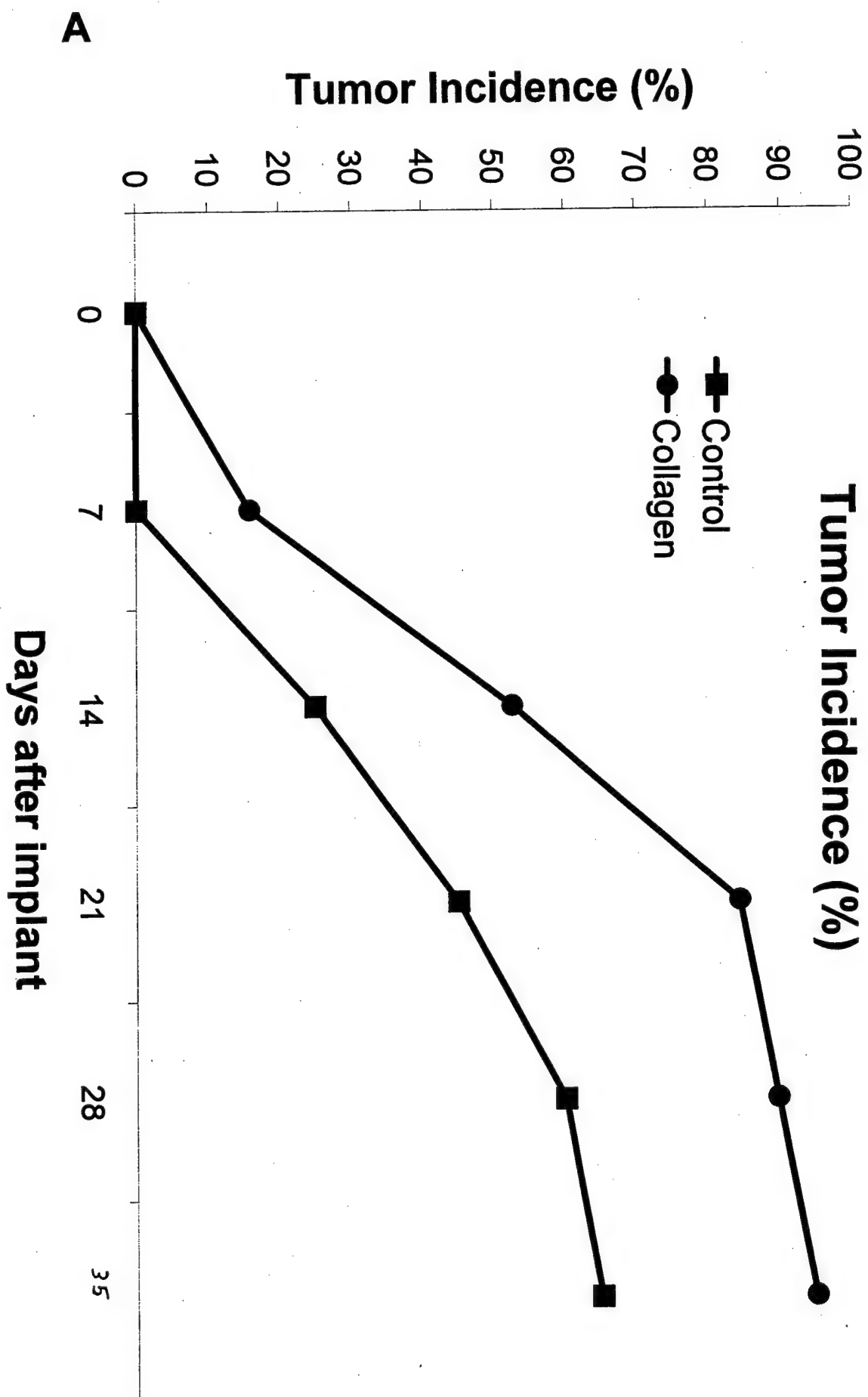


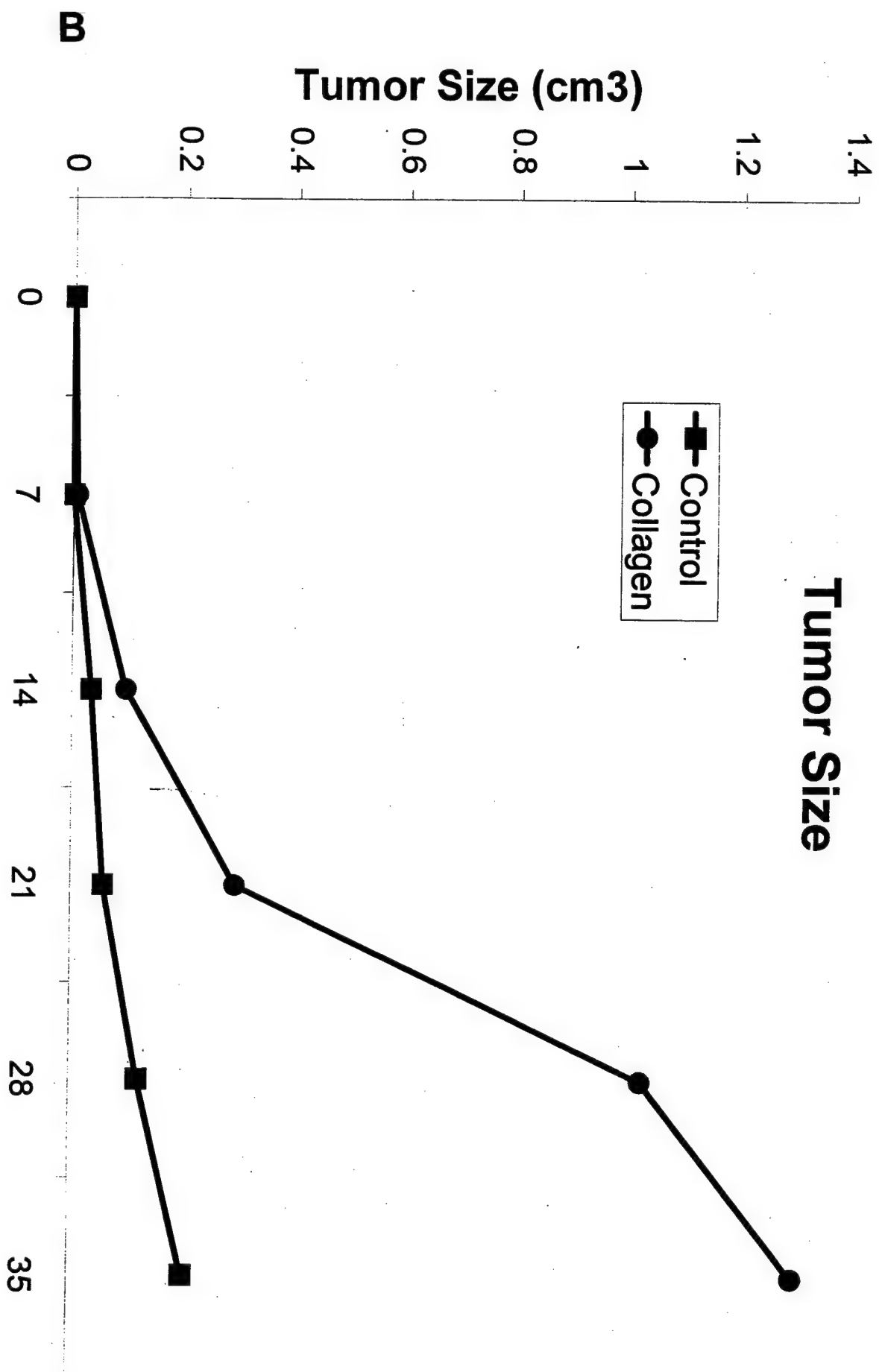




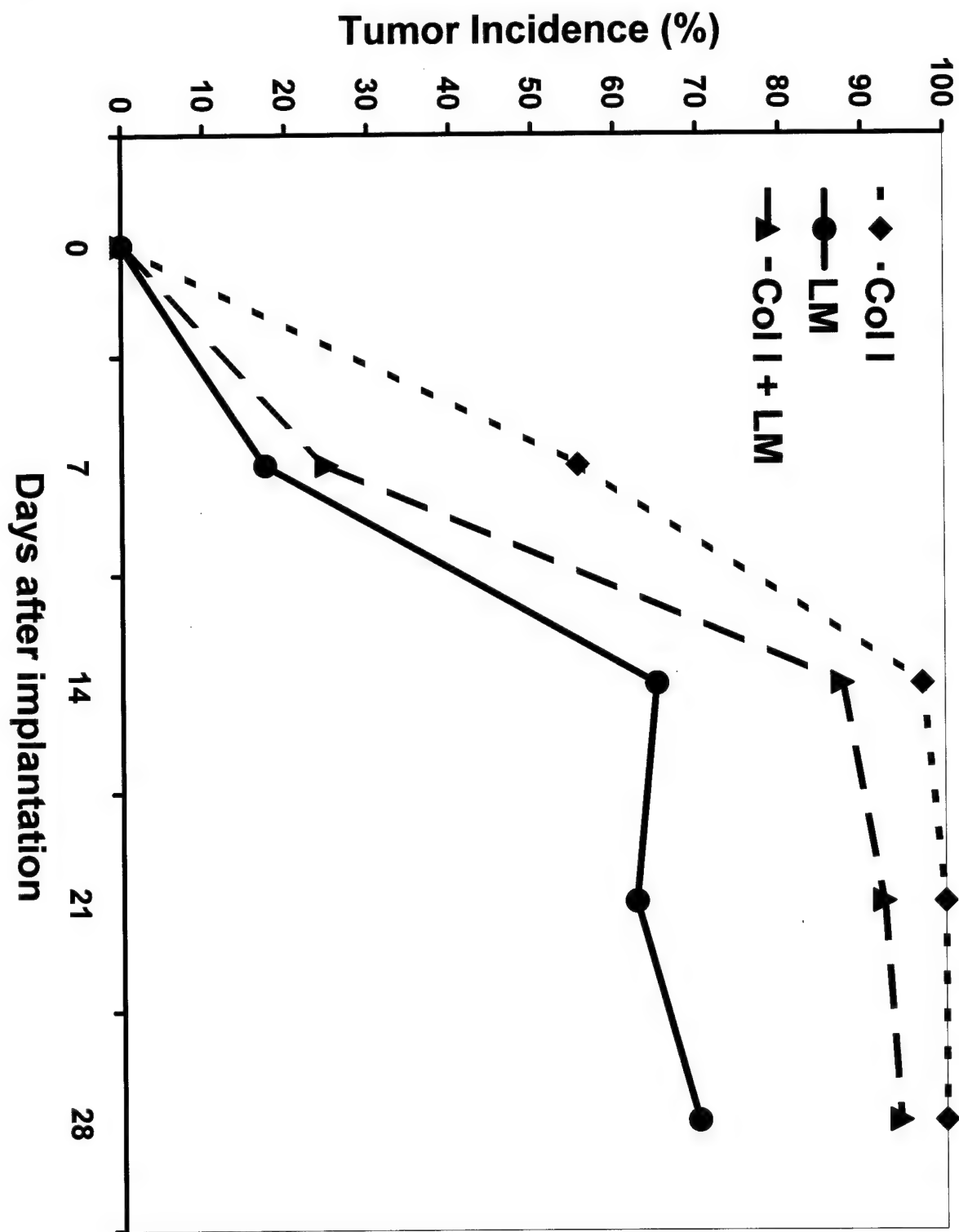






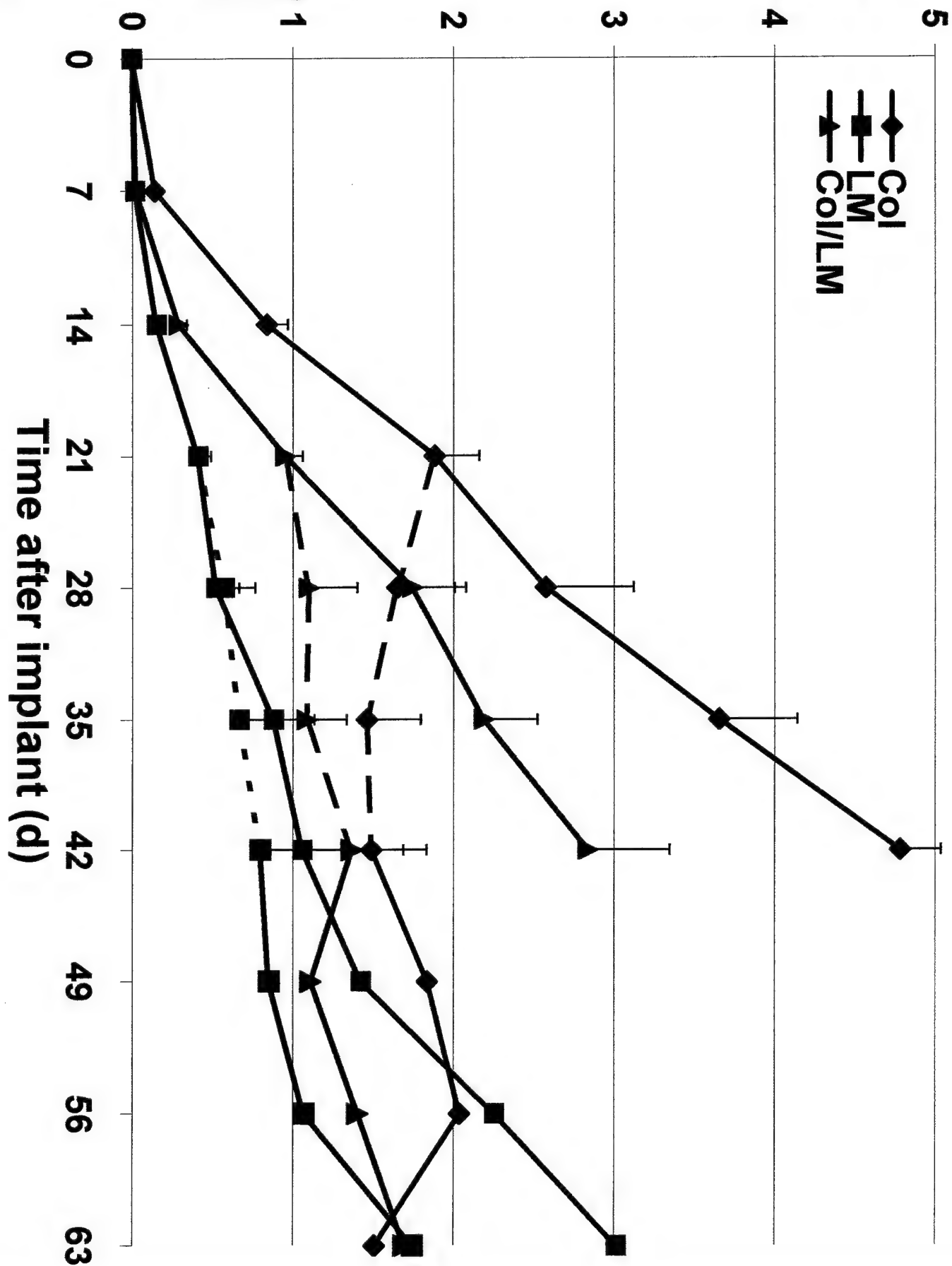


**A**



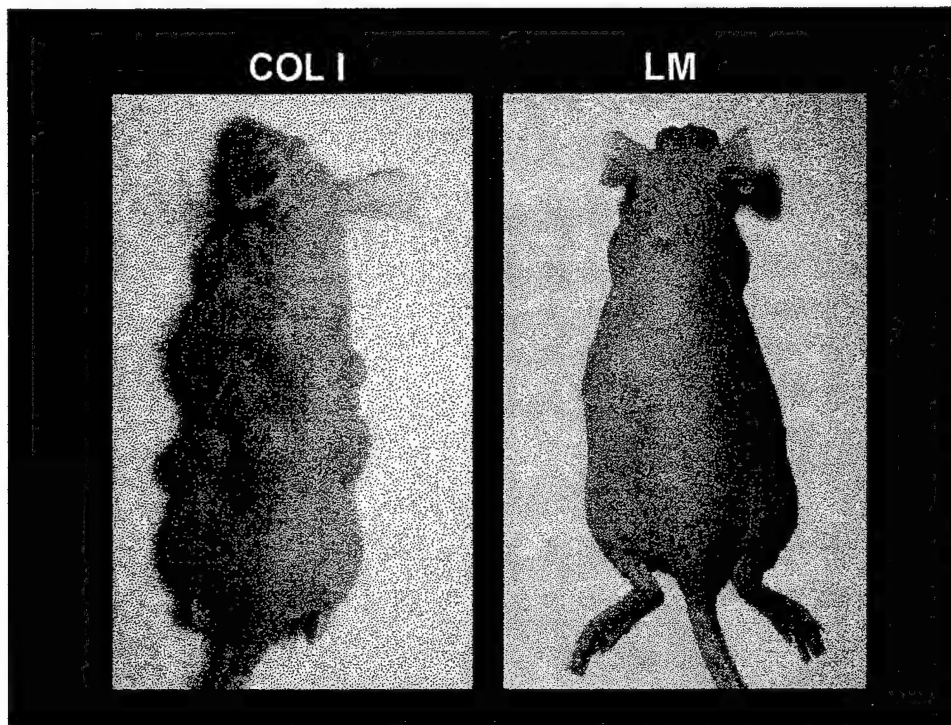
B

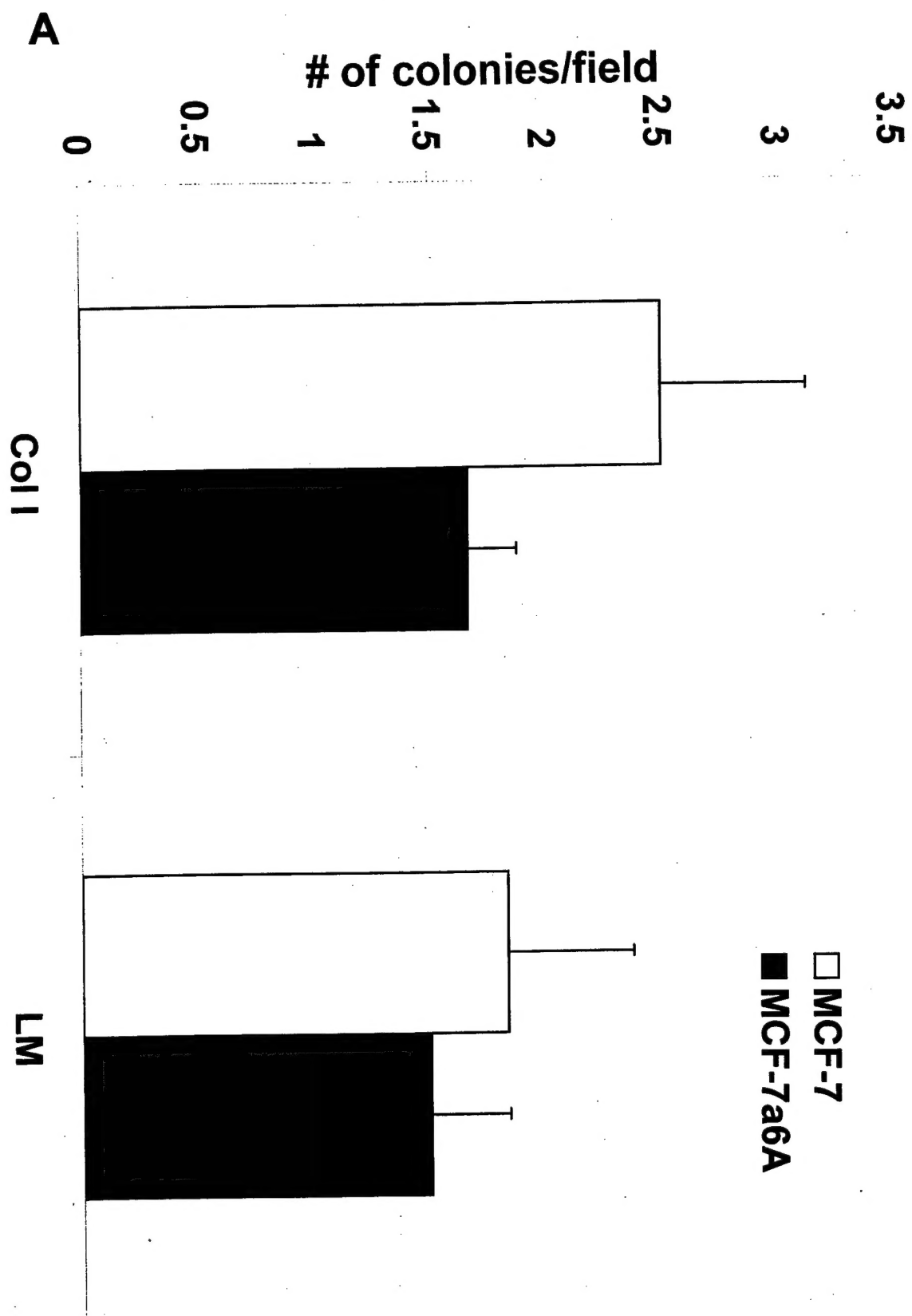
Average Tumor Volume/mouse (cm<sup>2</sup>)

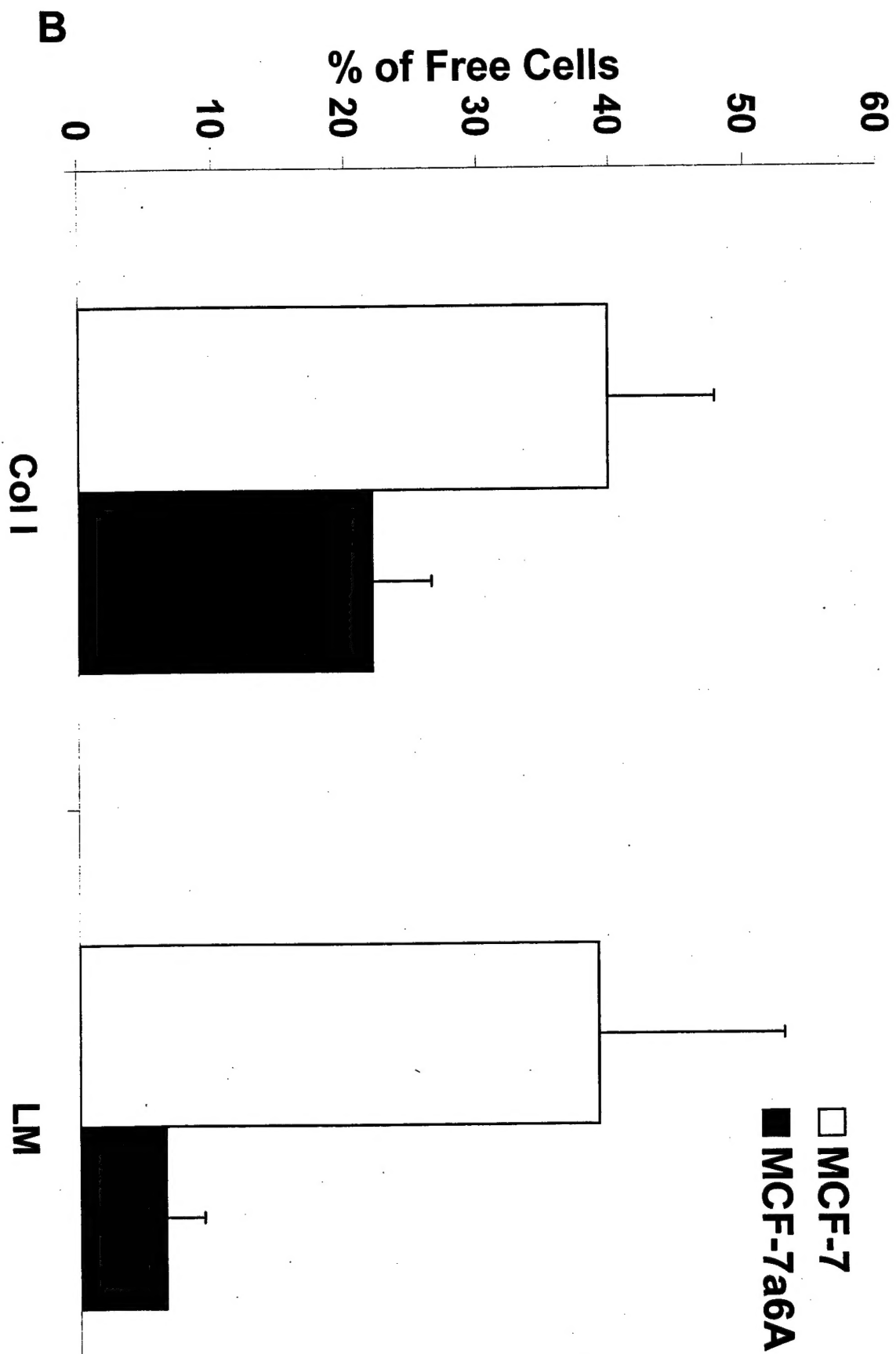


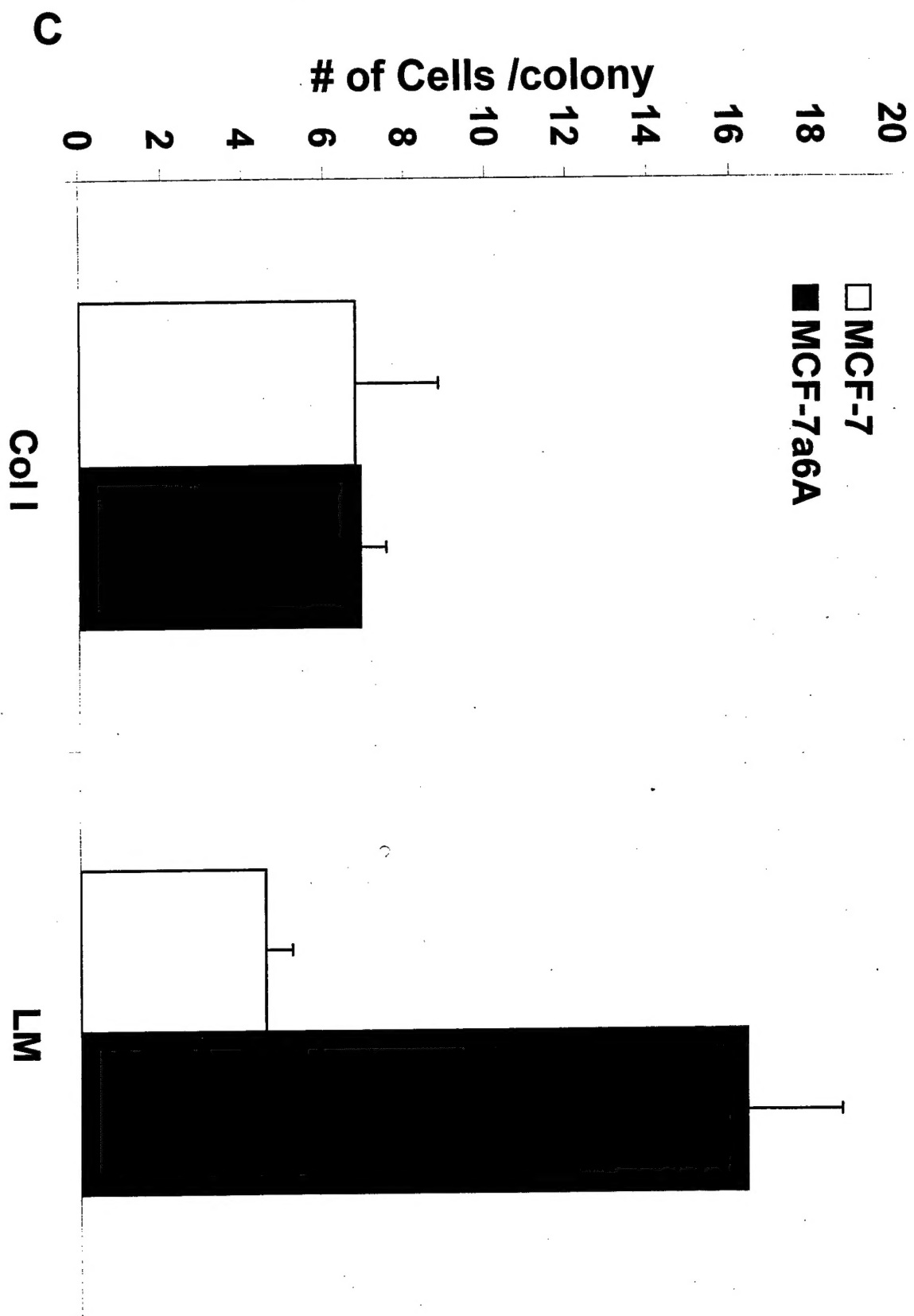


C









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